Papillomavirus and *c-myc* antigen expression in normal and neoplastic cervical epithelium

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SUMMARY Cervical punch biopsy specimens or brushings were collected from 33 patients with cervical human papilloma virus (HPV) infection, cervical intraepithelial neoplasia (CIN), or invasive cervical carcinoma, and from eight control patients with recent normal cervical cytology. Prostatic chippings obtained from six men with benign prostatic hypertrophy were used as further controls. Biopsy specimens and brushings were assayed by flow cytometry for *c-myc* oncogene antigen and papillomavirus antigen expression and rate of cell division (by measuring DNA content). Results obtained from analysis of specimens and brushings were similar in terms of *c-myc* antigen and total DNA content, but when the percentages of nuclei from biopsy and brush specimens staining positively with antibody to papilloma viral antigens were compared, brush specimens gave consistently higher percentages than biopsy specimens. More specimens from normal epithelium were *c-myc* antigen positive (five of eight, (63%)) than specimens from CIN II or III (two of 10, 20%), or invasive carcinoma (0%). No association was found between *c-myc* antigen expression and cell division. HPV antigen positive specimens were found to contain more dividing cells than negative specimens.

The association between human papillomaviruses (HPV) and cervical intraepithelial neoplasia (CIN) is well recognised, but whether the association is causal or casual remains uncertain.¹ The virus is believed to stimulate cell division within the squamous epithelium of the cervical transformation zone, and may cause arrest in the "S" phase (the phase of DNA synthesis) of the cell cycle.^{2,3} HPV DNA can be identified in 80-90% cases of CIN⁴ regardless of stage, but expression of viral antigens depends on epithelial differentiation and is therefore less common in CIN III than in CIN I.⁵

The *c-myc* oncogene is expressed in almost all differentiated cell types and the protein is thought to act as a transcriptional regulator.⁶ One group has shown that increased *c-myc* protein expression is closely associated with the entry of a cell from the G₀ or resting phase into the cell cycle, while almost complete shut off of *c-myc* expression accompanies the inhibition of proliferation associated with differentiation.⁷ Other workers, however, report that *c-myc* protein synthesis is independent of the cell cycle.⁸ Structural changes in the *c-myc* gene and abnormal expression of the *c-myc* product have been observed in numerous tumours, particularly B and T cell malig-

nancies.⁹ In the cervix, *c-myc* DNA amplification is reported to be associated with increasing severity of invasive carcinoma.^{10,11} Stage 1 and 2 carcinomas, in which *c-myc* mRNA overexpression is detected, are also more likely to relapse after treatment than carcinomas in which such overexpression is not detected.¹² In contrast, Hendy-Ibbs *et al* report a higher concentration of *c-myc* antigen in epithelial cells from normal cervix than in cells from CIN or from invasive cervical carcinoma.¹³

Modern flow cytometry makes it possible to use double staining techniques to assay cells or nuclei for two or more variables simultaneously. In this study a modification of the technique originally described by Watson *et al*¹⁴ was used. This enabled simultaneous oncoprotein or papillomaviral antigen detection and DNA assays to be carried out on cervical nuclei. The results obtained by this method provide further information about the associations between *c-myc* and papillomavirus antigen expression and cell cycle kinetics, and between the expression of these antigens and the development of cervical neoplasia.

Patients and methods

CONTROLS (Group 1)

Eight women undergoing minor gynaecological procedures (laparoscopic sterilisation, dilatation and

curettage, etc) who had had normal cervical cytology within the preceding three years acted as controls. Biopsy specimens from their cervical transformation zones were reported as containing no abnormality.

PATIENTS WITH CERVICAL HPV INFECTION OR CIN (Group 2)

Six patients with evidence of cervical HPV infection in the form of koilocytosis¹⁵ and 20 with CIN were studied. They had been referred to the Lothian Area Colposcopy Clinic on account of one or more suspicious cervical smear(s), or because of concern about the appearance of their cervix. Full colposcopic examination of the lower genital tract was performed and colposcopically directed punch biopsy specimens taken from all abnormal areas were visualised.

PATIENTS WITH INVASIVE CERVICAL CARCINOMA (Group 3)

Seven patients undergoing extended abdominal hysterectomy for invasive squamous cervical carcinoma were studied. Biopsy specimens were taken from the tumour and from adjacent cervical epithelium within 20 minutes of the specimen being removed from the patient.

CERVICAL BRUSH SPECIMENS

A Cytobrush (Medscand¹⁶) was used to obtain these specimens with the bristle end of the brush bent at an angle of about 50°, according to the method of Elias-Jones *et al.*¹⁷ The distal end of the brush was inserted just inside the external os and the brush rotated through 360° to obtain exfoliated cells from the whole of the transformation zone. The plastic holder was then cut in half and the brush placed in a sample tube containing 5 ml methanol. The sample was stored at room temperature until processed further. Satisfactory results were obtained from samples stored for up to four months.

CERVICAL BIOPSY SPECIMENS

Each biopsy specimen was divided; half was fixed for routine histopathological examination and half was immediately snap frozen on dry ice. A cryostat section 8 µm in thickness was cut from the centre of each snap frozen specimen and sections were prepared from the other half for routine histopathological assessment with a conventional haematoxylin and eosin stain.

CIN was graded according to recognised criteria¹⁸ and koilocytosis reported if present.¹⁵

The flow cytometer used was an EPICS 'C' (Coulter) with an argon ion laser emitting light at 488 nm and set at an output of 100–200 mW.

Cervical brush specimens were prepared for flow cytometry by first centrifuging the entire specimen (including the brush) at 700 × *g* for 10 minutes and then removing the methanol. After one wash in 0.01M phosphate buffered saline (PBS), pH 7.2, and a further centrifugation the sample was incubated according to a modification of the method of Hedley *et al.*¹⁹ It was suspended in 5 ml 0.5% pepsin solution (Sigma) in PBS, with the pH adjusted to 1.5 with hydrochloric acid at 37°C for 45 minutes; the brush was then agitated in the solution and discarded. The nuclei released by this procedure were centrifuged and the supernatant discarded. They were washed in PBS and respun.

Cervical biopsy specimens, thawed after the cryostat section had been cut, were minced and incubated in pepsin solution (as above). After 45–60 minutes the supernatant was drawn off and centrifuged at 700 × *g* for 10 minutes; the pellet was washed in PBS and respun at 700 × *g* for 10 minutes. Any clumps of nuclei were dispersed by syringing through a fine gauge needle (26g).

STAINING

Nuclear antigen

Nuclei were fixed in 1% formol saline in PBS containing 2% normal sheep serum (NSS) for 10 minutes at room temperature. They were centrifuged (all centrifugations were at 700 × *g* for 10 minutes) and resuspended in 20% NSS in PBS at a concentration of 10⁶ nuclei/ml for 20 minutes, and nuclear antigens were stained by a modification of the method of Elias-Jones *et al.*¹⁷ Nuclei were centrifuged and resuspended in primary antibody (200 µl per 10⁶ nuclei) overnight at 4°C (table 1). Primary antibodies were rabbit anti-bovine papillomavirus type 1 (Dakopatts),¹⁹ which recognises papillomavirus germ specific antigens and Mycl-6E10,²⁰ which recognises the *c-myc* product (p62). Primary and secondary antibodies were diluted in 2% NSS in PBS. Nuclei were then washed twice in 2% NSS in PBS and fluorescein isothiocyanate (FITC) conjugated secondary antibody (table 1) was added for 45 minutes at 4°C. Nuclei were washed twice in 2%

Table 1

Primary antibody	Specificity	Dilution	Source	Reference	Secondary antibody	Dilution	Source
habbit anti-bovine papillomavirus type 1	Papillomavirus genus-specific	1/50	Dakopatts	19	FITC conjugated sheep anti-rabbit immunoglobulin	1/30	Wellcome
Mycl-6E10	p62 <i>c-myc</i>	1/10	G Evan	21	FITC conjugated sheep anti-mouse immunoglobulin	1/40	Sigma

NSS in PBS and stored in 1% formol saline in the dark. Nuclei could be stored under these conditions for up to two weeks with no loss of staining.

Controls were prepared by substituting 2% NSS for primary antibody.

Chippings obtained from six patients at transurethral resection of prostate for benign prostatic hyperplasia were snap frozen and processed in the same way as the cervical biopsy specimens. They were then stained with antibody to papillomavirus antigens and used as controls for these experiments.

Nuclear DNA

Nuclei already stained with antibodies to nuclear antigens and FITC labelled conjugates, or nuclei immediately after pepsin digestion were stained with propidium iodide by the method of Deitch *et al*²² as previously described.²

FLOW CYTOMETRY – NUCLEAR ANTIGEN STAINING

A total of 40 000 nuclei were analysed from each sample at a flow rate of 400–500 nuclei/second. The nuclear fraction was selected by gating on a one-parameter histogram measuring log forward angle light scatter (LFLS), and the percentage of nuclei showing green fluorescence greater than the background level was recorded. Fluorescent signals were simultaneously acquired from a two-parameter histogram measuring forward angle light scatter against 90° light scatter using a bit map around the nuclear population. The results from both histograms differed by less than 2%, and all results presented are those acquired by gating on LFLS signals.

FLOW CYTOMETRY – NUCLEAR DNA STAINING

Nuclei were run at a rate of 150/second until 20 000 stained nuclei had been counted. Chicken red blood cells (CRBC), stained with propidium iodide as above, were run with each sample as an internal control, the DNA content of these cells being equivalent to 35% of that of a human diploid cell.²² Human peripheral blood lymphocytes were stained and run with CRBC as an additional DNA control at the start of each run.

The data obtained were subjected to parametric analysis with the EPICS PARA I program, which determined the proportion of nuclei in each phase of the cell cycle.

Results

Between 10^6 and 5×10^6 (mean 2×10^6) nuclei were obtained from a biopsy specimen, and between 0.5×10^6 and 6×10^6 (mean 1.6×10^6) from a brush specimen.

Propidium iodide staining gave very similar results from both brushings and biopsy specimens; if the percent S + G₂ + M phase values from all individual patients were compared the difference was never more than 3%. When *c-myc* oncogene protein was measured results from biopsy specimens and brushings from the same patients did not differ by more than 2%. Furthermore, if the biopsy specimen from an individual patient was antigen positive, the brushing was also positive (and vice versa).

The percentage of papillomavirus antigen positive nuclei in biopsy specimens and brushings from the same patient varied considerably.

C-MYC ANTIGEN STAINING

The number of specimens in each histological category staining positively with the anti-*c-myc* antibody Mycl-6E10 is shown in table 2. The percentage of specimens from normal cervical epithelium that were *c-myc* antigen positive was higher (63%) than the percentage of *c-myc* positive specimens from CIN II or CIN III (20%) or invasive carcinomas (0%). These differences were not significant by Fisher's exact probability test ($p < 0.05$), although the difference between normal and invasive carcinoma does reach significance at the $p < 0.2$ level. The range of percentages of nuclei staining positively with Mycl-6E10 (in the positive specimens) is also shown. If *c-myc* positive specimens in each histological group were compared the percentage of nuclei in each specimen staining positively did not vary by more than 2.3% (table 2).

The percentage of nuclei in the S + G₂ + M phases of the cell cycle in *c-myc* antigen negative and *c-myc*

Table 2 *c-myc* antigen in biopsy specimens and brushings from normal and abnormal cervical epithelium

Cervical histology	Number of specimens <i>c-myc</i> antigen positive (%)			Mean (and range) per cent nuclei expressing <i>c-myc</i> antigen (within <i>c-myc</i> positive specimens)
	Brushings	Biopsies	Total*	
Normal (n = 8)	4/6 (67)	3/4 (75)	5/ 8 (63)	3.5 (2.6–5.4)
Koilocytosis alone (n = 5)	0/2 (0)	1/4 (25)	1/ 5 (20)	2.0
CIN I (n = 10)	2/5 (40)	3/6 (50)	5/10 (50)	4.3 (3.0–7.4)
CIN II (n = 5)	1/3 (33)	0/3 (0)	1/ 5 (20)	2.4
CIN III (n = 5)	0/2 (0)	1/5 (20)	1/ 5 (20)	3.7
Invasive carcinoma (n = 7)		0/7	0/ 7 (0)	

*Seven patients had both brushings and a biopsy specimen processed for flow cytometry.

Table 3 Cell cycle analysis and c-myc antigen expression

Cervical histology	Per cent (SEM) nuclei in S + G ₂ + M phases	
	c-myc antigen negative specimens	c-myc antigen positive specimens
Normal	22 (3.8) (n = 3)	18 (2.9) (n = 5)
Koilocytosis alone	32 (3.1) (n = 4)	31 (n = 1)
CIN I	33 (3.7) (n = 5)	31 (3.5) (n = 5)
CIN II	32 (3.1) (n = 4)	32 (n = 1)
CIN III	32 (2.7) (n = 4)	30 (n = 1)

positive specimens is shown in table 3. Within each histological group, there was no difference in the percentage of nuclei in the S + G₂ + M phases when c-myc antigen negative and c-myc positive specimens were compared.

PAPILLOMAVIRUS ANTIGEN STAINING

The results obtained from staining cervical nuclei with an antibody to papillomavirus antigens are shown in table 4. The proportion of specimens exhibiting positive staining with the antibody ranged from 38% (normal cervixes) to 83% (koilocytosis alone). A similar proportion of biopsy specimens and brushings from CIN with (67%) and without (75%) koilocytosis stained positively with the antibody. Nine patients had both a biopsy specimen and a brush sample tested for the presence of papillomavirus antigens. In seven patients both the biopsy and the brush specimens stained positively; in one patient both were negative; while in one patient with a cytologically, colposcopically, and histologically normal cervix the biopsy gave a negative result while the brushing stained positively with the antibody to papillomavirus antigens.

When the percentage of nuclei from brush and biopsy samples staining positively with the antibody were compared brush samples gave consistently higher percentages than biopsy specimens (table 1). Viral antigen positive brush samples from the different histological groups contained similar percentages of positive nuclei; similarly, there was no difference

Table 5 Cell cycle analysis and papillomavirus antigen expression

Cervical histology	Per cent (SEM) nuclei in S + G ₂ + M phases	
	Antigen negative specimens	Antigen positive specimens
Normal	21 (3) (n = 5)	27 (4)* (n = 3)
Koilocytosis alone	24 (n = 1)	32 (n = 5)
CIN without koilocytosis	21 (4) (n = 2)	34 (4) (n = 6)
CIN with koilocytosis	29 (2) (n = 3)	30 (4) (n = 6)

*The patient with a biopsy specimen negative for HPV and one HPV positive brush specimen is included in the antigen positive group.

between the HPV antigen positive biopsy samples from the different histological groups.

The percentage of nuclei in the S + G₂ + M phases of the cell cycle in HPV negative and positive specimens is shown in table 5. Within each group except CIN with koilocytosis, HPV positive specimens contained more dividing cells than HPV negative specimens. The differences were not significant (Mann Whitney U test) owing to the small numbers in the HPV negative groups.

The results obtained from staining six specimens of prostatic chippings obtained at transurethral resection of prostate are shown in table 4. No positive staining was obtained with antibody to papillomavirus antigens in these specimens.

No HPV antigen expression was detected in seven cases of stage Ib cervical carcinoma. This was not unexpected as antigen expression is known to decrease as the severity of cervical neoplasia increases.⁵ HPV antigen was detected in two of four biopsy specimens from normal epithelium adjacent to invasive carcinomas, and in two of three biopsy specimens from dysplastic epithelium adjacent to invasive carcinomas. This was also anticipated as Kurman *et al*⁶ reported antigen expression in CIN I and II adjacent to CIN III which was antigen negative; while Ferenczy *et al*²⁴ found the presence of HPV DNA in biopsy specimens taken from normal skin adjacent to treated anogenital warts.

Table 4 Papillomavirus antigen expression in biopsy specimens and brushings from normal and abnormal cervical epithelium

Cervical histology	Number (%) specimens papillomavirus antigen positive			Mean (range) per cent nuclei papillomavirus antigen positive (within antigen positive specimens)	
	Brushings	Biopsies	Total*	Brushings	Biopsies
Normal (n = 8)	2/4 (50)	2/6 (33)	3/8 (38)	23 (20-26)	4 (3-5)
Koilocytosis alone (n = 6)	3/3 (100)	4/5 (80)	5/6 (83)	24 (8-35)	5 (3-11)
CIN without koilocytosis (n = 8)	4/4 (100)	4/6 (67)	6/8 (75)	18 (4-26)	4 (3-9)
CIN with koilocytosis (n = 9)	4/4 (100)	5/8 (63)	6/9 (67)	15 (6-28)	6 (3-13)
Benign prostatic hypertrophy (n = 6)		0/6	0/6		

*Nine patients had both brushings and a biopsy specimen processed for flow cytometry.

Discussion

COMPARISON OF CERVICAL BRUSHINGS AND BIOPSY SPECIMENS

Several authors have advocated the use of cervical brushes for obtaining specimens for routine cytopathology,¹⁶ for HPV DNA extraction,²⁵ and for flow cytometric analysis.¹⁷ The data obtained in the present study show that cervical brush specimens give very similar results to cervical biopsy specimens when analysed for DNA content and for expression of the *c-myc* oncogene product, and thus offer a non-invasive alternative to biopsy, causing less discomfort to the patient and less likelihood of changing the natural history of cervical lesions. The results obtained concerning HPV antigen expression in brushings, however, did differ in that the percentage of positive nuclei within positive samples was higher in brushings than in biopsy specimens. This is presumably a sampling effect as a brush removes predominantly superficial epithelial cells which are more likely than deeper cells to be antigen positive,⁵ while a biopsy normally removes the full epithelial thickness as well as some underlying stroma. Despite this, when biopsy and brush samples were taken from the same patient, the two samples gave the same result (either HPV positive or negative) in eight of nine patients.

ONCOPROTEIN STAINING

Interestingly, the percentage of specimens from normal cervical epithelium which were *c-myc* antigen positive was higher than the percentage from CIN II or III, or invasive carcinomas, although not significantly so. *c-myc* DNA amplification has been found in more advanced cervical carcinoma,^{10,11} and increased concentration of *c-myc* RNA are associated with early clinical relapse in stages I and II carcinomas.¹² In contrast, a higher concentration of *c-myc* antigen expression in normal cervical epithelium than in neoplastic epithelium has been reported in wax embedded tissue¹³ which corresponds to the findings reported here. Increased expression of *c-myc* antigen in normal, relative to dysplastic epithelium, may be due to an increased turnover of the protein in dysplastic cells, as it has a short half life in stimulated cells.^{8,26} It has been suggested that the *c-myc* product may be especially susceptible to proteolysis in neoplastic cells during the preparation for the assay, or that Mycl-6E10 may recognise an epitope of some other nuclear associated proteins.¹³ Another antibody recognising a different region of the protein, however, gave the same results.¹³ Mycl-6E10 therefore probably does recognise the *c-myc* product. The clear difference in *c-myc* antigen content between normal and abnormal cervical epithelium has obvious diagnostic potential, and automated cervical screening could perhaps

include this variable, together with others such as nuclear:cytoplasmic ratio²⁷ and DNA content.²⁸

HPV ANTIGEN STAINING

The finding of HPV antigen expression in 38% of otherwise normal cervixes is consistent with reports published elsewhere that between 11%²⁹⁻³¹ and 50%³² of otherwise normal cervixes contain HPV DNA. The proportion of dysplastic biopsy specimens containing HPV antigen positive nuclei (71%, table 4) is higher than is generally reported for immunohistochemical studies of paraffin wax sections.⁵ HPV DNA, however, can be isolated from 70%³³ to 85%³⁴ of dysplastic cervical biopsy specimens. The higher concentration of antigen expression in CIN reported here compared with that of immunohistochemical studies may be due to the fact that flow cytometry permits the rapid analysis of many thousands of nuclei. It is also possible that the method of preparation of nuclei exposes the viral antigens in a way which makes them more accessible to antibody.

It has been postulated that cells infected with HPV behave initially as though stimulated by a non-specific mitogen and that in a semipermissive epithelium many cells remain in a prolonged S or G₂ phase.³ We have previously shown an increase in cellular proliferation in koilocytic lesions in the absence of CIN², and the demonstration here of an increased proportion of nuclei in the S + G₂ + M phases in HPV antigen positive specimens is further evidence to support the hypothesis that HPV infection stimulates increased cell division.

Thus evidence is provided to support the use of the cervical brush as a non-invasive alternative to punch biopsy in obtaining specimens suitable for flow cytometric analysis. We believe that flow cytometric assay of *c-myc* antigen content and cellular DNA content should be further investigated as a possible diagnostic tool for screening purposes. Our data confirm the reported finding of Hendy-Tibbs *et al*,¹³ that *c-myc* antigen expression is higher in normal than in neoplastic cervix. Further work using unfixed cells will be necessary to discover whether this finding has relevance to the *in vivo* role of the *c-myc* oncogene in proliferation control. The present study supports our earlier contention² that HPV stimulates epithelial cell division, and we believe that HPV may be a carcinogenic agent simply by providing a greater number of opportunities for mutations and neoplastic change.

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