Papers

Endocervical carcinoma and precursor lesions: *c-myc* expression and the demonstration of field changes

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Abstract

The monoclonal antibody Myc 1-6E10 was used in an immunocytochemical technique to examine the expression of the *c-myc* oncogene in normal endocervices and those with glandular intraepithelial neoplasia and invasive malignancy. Eleven of 14 normal endocervical biopsy specimens did not express the gene, while three showed nuclear, or light basal cytoplasmic localisation of the gene product, or both. All but one of 14 cases of low and high grade glandular intraepithelial neoplasia, and all 17 cases of invasive adenocarcinoma, showed a pan-cellular pattern of immunostaining. Of considerable additional interest was the demonstration of field changes in morphologically normal glandular epithelium in those biopsy specimens with concurrent cervical glandular intraepithelial neoplasia or adenocarcinoma. This was manifest as increased concentrations of *myc* proteins compared with normal tissues.

These results further support the role of the *c-myc* gene in oncogenesis, and in the light of field changes, suggest possible difficulties in the clinical management of this group of patients.

The incidence of adenocarcinoma of the uterine cervix in women under 30 years of age has increased in the past 10 years, both in absolute terms¹ and in the proportion of total cervical malignancies that these tumours comprise.^{2,3} The natural history of these tumours has been less well investigated than that of their squamous counterparts. Although some evidence suggests that adenocarcinoma at this site can arise de novo,⁴ this might be explained by the underdiagnosis of in situ lesions.^{5,6} The terminology used for such in situ lesions is somewhat confusing. We have chosen to use the term glandular intraepithelial neoplasia (GIN), and to subdivide these histologically into low or high grade lesions. Others use the term cervical glandular atypia; these lesions are also subdivided into low or high grade, the latter alternatively referred to as adenocarcinoma in situ.⁷ The biological behaviour of GIN remains unclear and the diagnosis of such

lesions difficult. These difficulties conspire to make the clinical follow up of cases problematical.

The diagnostic use of immunocytochemistry in this area has been little studied. Anti-epithelial membrane antigen staining is identical in high grade glandular intraepithelial neoplasia and invasive adenocarcinoma,⁸ but differential expression of human milk fat globulin antigen in GIN and the normal endocervix does seem to occur.⁹ Other markers have also been studied, including those directed against intermediate filaments and carcinoembryonic antigen.¹⁰ An attempt has been made to correlate silver nucleolar organiser region (AgNor) counts in GIN and invasive adenocarcinoma.¹¹

The examination of oncogene expression in a variety of malignant and pre-malignant states has proved to be of variable diagnostic use. Oncogenes are highly conserved regions of the normal genome. A change in the coding or controlling regions of these genes has been implicated in the pathogenesis of neoplasia. The human *c-myc* oncogene is the cellular homologue of the avian *v-myc* gene found in some leukaemogenic retroviruses. The gene has been shown to have an important role in early embryogenesis,¹² the control of cell growth,¹³ cellular differentiation^{14,15} and tissue repair.¹⁶

Expression of the gene is therefore considered to be a major component of the regulatory processes associated with cell proliferation and differentiation. This view is reinforced by the demonstration of gene amplification, or an increase in the level of the gene product p62^{c-myc} in a variety of pathological states in which there is a perturbation of these processes.¹⁷⁻¹⁹

No attempt has been made to examine the expression of the *c-myc* gene in malignant and pre-malignant lesions of the endocervix, although a number of studies have looked at gene expression in cervical intraepithelial neoplasia and in squamous carcinoma at this site.²⁰⁻²³

Methods

Archival paraffin wax embedded tissue blocks were obtained from 45 patients who underwent cone or colposcopic biopsy or hysterectomy.

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Figure 1 Normal endocervical glands showing no expression of myc protein.

Figure 2 High grade GIN showing pan-cellular myc protein distribution.

Figure 3 Invasive endocervical carcinoma showing pan-cellular myc protein distribution.

Figure 4 Morphologically normal endocervical glands close to an area of GIN showing nuclear and basal cytoplasmic myc protein distribution.

tomy. To increase the numbers in the study tissue blocks were obtained from four other centres in the United Kingdom. Of these 45 patients, 14 had a normal endocervix and served as external controls. The remaining 31 patients had either GIN or invasive adenocarcinoma. Using standard histological criteria⁷ glandular intraepithelial neoplasia was subclassified as low grade (four cases) and high grade (10 cases). Those with invasive adenocarcinoma (17 cases) comprised a uniform population of well differentiated (five cases), moderately differentiated (five cases), and poorly differentiated (seven cases) tumours. More precise clinical details were not available, largely because of the high proportion of referred material.

Sections were cut at 4 µm and placed on glass microscope slides and oven dried at 50°C overnight. Sections were dewaxed and rehydrated using xylene and alcohols. Endogenous peroxidase was blocked by a 10 minute incubation in 0.5% hydrogen peroxide in methanol. The sections were washed briefly and incubated for two hours in Myc 1-6E10 (Cambridge Research Biochemicals, Eng-

land), diluted 1 in 10 000 in TRIS-buffered saline with 1% bovine serum albumin and 0.25% Triton X-100 (pH 7.3). After further washing, biotinylated sheep anti-mouse immunoglobulin was applied to the sections at a dilution of 1 in 100 and incubated for 60 minutes. After additional washes, incubation in Vectostain ABC reagent (Vector Laboratories, England) was performed for one hour, followed by a final incubation in diaminobenzidine (100 mg in 100 ml 0.5% hydrogen peroxide solution) for 10 minutes. The sections were washed in tap water and counterstained in Harris's haematoxylin for 60 seconds.

All washes, unless specifically stated, were performed using TRIS-buffered saline containing 1% bovine serum albumin, and incubations were at room temperature.

The specificity of Myc-1-6E10 has been investigated and its immunoreactivity found to be annulled by addition of the peptide used as immunogen in the production of the antibody.²⁴

Results

Of the 14 normal endocervical specimens, 11 cases were completely unstained, indicating that *c-myc* oncogene was not expressed (fig 1). Three cases showed weak positive staining of the nucleus, and of these, two cases showed additional weak positive staining of the basal cytoplasm. In none of these cases was positive staining of the apical cell cytoplasm shown (table 1).

Table 1 Immunostaining pattern using Myc 1-6E10

Tissue classification	No of cases	Staining pattern		
		Nuclear	Cytoplasmic	Negative
Normal endocervix	14	3	2	11
GIN:				
Low grade	4	3	3	1
High grade	10	10	10	0
Invasive adenocarcinoma	17	17	17	0

Table 2 Immunostaining pattern in histologically normal endocervical glands in patients with concurrent GIN or adenocarcinoma

	No of cases	Staining pattern		
		Nuclear	Basal cytoplasm	Apical cytoplasm
Controls	14	3	2	0
"Normal" glands concurrent with GIN	12	9	6	0
"Normal" glands concurrent with adenocarcinoma	9	9	6	0

All but one of the 14 cases of low and high grade GIN showed pan-cellular *c-myc* distribution, with weak positive staining of the cell nucleus, together with positivity of the basal and apical cell cytoplasm (fig 2). There was no distinction, based on the cellular distribution of protein, between low and high grade GIN, although the single non-staining case was histologically low grade.

All 17 cases of invasive endocervical carcinoma showed a pan-cellular distribution of *c-myc* (fig 3). There was no difference in cellular distribution between tumours of varying degrees of differentiation, but poorly differentiated tumours tended to stain less intensely than better differentiated tumours. This may represent a lower level of gene expression in these tumours, but no formal attempt has been made to quantify the concentrations of *myc* proteins in these tumours.

Of particular interest to us was the staining pattern observed in morphologically normal endocervical glands adjacent to areas of glandular intraepithelial neoplasia or carcinoma. Of 12 cases (out of the original 14) of GIN, in which morphologically normal glands were included in the tissue section, nine cases showed positive nuclear staining (fig 4), and of these, six showed additional positive basal cytoplasmic staining (table 2). This staining pattern was seen in those with both low and high grade GIN elsewhere in the biopsy specimen. Nine of the cases of invasive adenocarcinoma included morphologically normal endocervical glands in the same tissue section. All nine cases showed positive nuclear staining, and six of these showed basal cytoplasmic staining (table 2). In none of the normal glands was the pan-cellular pattern of immunostaining characteristic of glandular intraepithelial neoplasia or carcinoma seen.

Discussion

The expression of the *c-myc* gene in cervical squamous carcinoma and in situ squamous lesions of the cervix has been an area of intensive study.²⁰⁻²³ Gene amplification has been reported with a wide range of recorded incidence, some studies showing no evidence of amplification,²⁵ while others showed amplification in about 90% of all tumours in a sizeable group of Mexican patients.²³ Such results may reflect racial differences, this is unlikely, and indeed substantial differences have been documented among Caucasian patients of western origin.^{20, 25}

There is a similar lack of concordance with respect to *myc* protein concentrations in

cervical squamous carcinoma. In one study 25 of 72 tumours showed gene overexpression, with levels 4–20-fold that of controls;²⁰ others have shown greater concentrations of oncoprotein in normal cervical biopsy specimens than in carcinomas.^{22, 26}

It is against this confused background that our own results are presented. We have shown substantial expression of the *c-myc* gene in glandular intraepithelial neoplasia and in malignant endocervical cells. Furthermore, *myc* immunostaining seemed to be a powerful discriminator between normal cervical glandular epithelium and epithelium showing intraepithelial changes or overt malignant change.

Normal endocervical epithelial tissues showed only very occasional evidence of *myc* expression, and in no instance was staining of the apical cell cytoplasm observed. Epithelial tissues showing glandular intraepithelial neoplasia or malignant change invariably expressed the *myc* gene, with a pan-cellular distribution of *myc* product. Apical cytoplasmic *myc* localisation thus seemed to be specific for GIN and invasive adenocarcinoma at this site, and *myc* expression alone was strongly correlated with such changes in cell phenotype.

These features may be of diagnostic value in distinguishing low grade glandular intraepithelial neoplasia from normal epithelium, although the clinical importance of such low grade lesions is as yet imprecisely defined.⁷

Immunostaining for *myc* proteins does not permit discrimination between glandular intraepithelial neoplasia and malignancy, or between low and high grade GIN. The pattern of staining in all these conditions is identical, with a pan-cellular distribution of *myc* protein.

In addition to the expected nuclear siting of *myc* protein, we have shown considerable location of *myc* product in the endocervical cell cytoplasm. This cytoplasmic location has been noted in a variety of other tissues, and the explanation of such localisation is still imperfectly understood.^{27, 28} Although inadequate tissue fixation can produce such a distribution,²⁹ this does not necessarily indicate that such localisation is invariably a result of poor fixation, and a variety of defective or aberrant cellular mechanisms have been proposed.³⁰

Our observations on morphologically normal endocervical glands in those biopsy specimens with concurrent GIN or carcinoma are potentially of considerable importance. The immunostaining pattern obtained was in some respects intermediate between the largely negative normal endocervix in those women without concurrent disease and the pan-cellular pattern of immunostaining obtained with established glandular intraepithelial neoplasia or invasive malignancy. The number of cases in this area is unavoidably low, reflecting the difficulty in obtaining unequivocal examples of glandular intraepithelial neoplasia, particularly those histologically low grade. The pattern of immunostaining does, however, strongly suggest a field change effect in these morphologically normal glands that is a biological perturbation of cellular function in

epithelium with none of the features of glandular intraepithelial neoplasia. As all those with concomitant malignancy showed this aberration of *myc* expression in adjacent morphologically normal glandular epithelium, these changes may occur more often in such patients than in those with glandular intraepithelial neoplasia alone. These findings are further supported by additional observations of our group reported elsewhere,¹¹ showing substantially raised AgNor counts in such morphologically normal glands compared with an external control group. If substantiated, these observations raise important questions about the laboratory diagnosis of completeness of excision at cone biopsy and the follow up of patients with pre-malignant glandular lesions using conventional morphological criteria. It has been suggested that cone biopsy may be inadequate treatment for high grade intraepithelial neoplasia.³¹

That oncogene expression should in some way correlate with tumour behaviour is an attractive hypothesis. Overexpression of the *myc* gene in cervical squamous carcinoma has been shown to increase significantly the incidence of early disease relapse following surgery,²⁰ but, interestingly, does not seem to correlate directly with clinical disease stage.³² *Myc* overexpression has been detected in early cervical cancers, and it has been suggested that this may provide a means of identifying patients at high risk of early clinical recurrence of their disease.²¹

Our results support the view that the *c-myc* gene may have a role in the malignant transformation of glandular epithelium of the cervix, either alone or possibly in concert with other cellular oncogenes and viral antigens. Some evidence exists for the synergistic action of the *c-myc* gene, human papillomavirus, and herpes simplex virus in the cellular processes leading to cervical intraepithelial neoplasia and cervical squamous carcinoma.²⁵

The demonstration of field changes in morphologically normal endocervical tissue raises the possibility of underdiagnosis of glandular intraepithelial neoplasia, particularly as routine cytology is known to be poor at detecting atypical glandular cells.³³ It is for this reason that the diagnostic use of *myc* immunostaining of cytological preparations to detect low grade GIN is currently under review.

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