Technical method

Cervical brush biopsy specimens suitable for DNA and oncoprotein analysis using flow cytometry

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Carcinoma of the cervix is one of the few cancers for which effective screening could be carried out for most of the population at risk. Recent official recommendations in the United Kingdom suggest that cervical smears should be carried out at 5 yearly intervals. The past decade, however, has seen a dramatic increase in the incidence of abnormal smears, especially in young women.¹⁻³ This must generate a need for more smears, not only during follow up after treatment but also for initial diagnosis. Increasing the number of cervical smears/person would overwhelm existing analysis resources: the requirement for automated analysis is obvious.

Flow cytometry enables physical and biochemical measurements to be made on a cell to cell basis, and, typically, up to 5000 cells can be analysed/second. Recently reports have been published in which DNA and oncoproteins have been measured simultaneously not only in tissue culture cells⁴ but also in nuclei extracted from paraffin embedded biopsy specimens.^{5,6} These methods used flow cytometric quantitation with fluorescence staining techniques, using monoclonal antibodies for the oncoproteins and propidium iodide for DNA.

This paper shows that brush biopsy specimens of the cervix yield sufficiently good quality material to enable the simultaneous oncoprotein and DNA assay to be carried out. Specimens so collected can be sent through the regular mail, and we will be exploring the possibility that the technique can be used as an automated screening procedure for cervical cancer.

Material and methods

TISSUE SAMPLING

Patients attending the colposcopy clinic in the univer-

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sity department of obstetrics and gynaecology, Addenbrooke's Hospital, were included in the study. The upper vagina and cervix were gently swabbed to remove excess mucus. The cervix was then gently brushed with a Cyto-Brush, a batch having been kindly donated by Medscand (Malmo, Sweden). To facilitate sampling the bristle end of the brush was bent over at an angle of about 50° (Fig. 1), enabling it to be used similarly to a conventional spatula. Cells trapped in the bristles were examined by conventional cytology after wiping one side of the brush over a glass slide. The plastic holder was then cut in half and the brush was placed in a sample tube containing 5 ml methanol (Fig. 1). Fig 2 shows a photograph of a sample being taken from the endocervix.

FLOW CYTOMETRY

Preparation of nuclei The brush was agitated in the methanol to shake out the fixed cells. The sample was then centrifuged at 200 g and the methanol removed. After one wash in phosphate buffered saline (pH 7.4) and a further centrifugation the sample was resuspended in 2 ml of pepsin solution (Sigma) at a concentration of 5 mg/100 ml, pH 1.9. After incubation for 45 minutes at 37° the nuclei released by cytoplasmic digestion were centrifuged and the pepsin containing the supernatant was removed. The pellet was resuspended in 6 ml phosphate buffered saline and one ml aliquots were placed into six 1.5 ml Eppendorf tubes, centrifuged, and the phosphate buffered saline removed.

Simultaneous oncoprotein and DNA staining The oncoprotein quantitated in these studies (p62^{c-myc})

Fig. 1 Cyto-Brush after bending bristle section (left) and immersion in methanol in sample tube (right).

Fig. 2 Endocervical sampling in a case of CIN 3 disease.

was the product of the *c-myc* gene. A monoclonal antibody Myc 1-6E10 was raised to a synthetic peptide predicted from the base sequence of the cloned gene.⁸ Four nuclear pellets were resuspended in 20 μ l of diluted antibody to probe the oncoprotein. The dilutions used were 1/10, 1/3·16, 1/100, and 1/316, and after incubation for 45 minutes all samples were centrifuged and the supernatants removed. The four samples treated with the antibody and one of the controls were then incubated with 10 μ l of fluorescein isothiocyanate conjugated rabbit antimouse Ig (Dako, Denmark) diluted 1/50. After one hour all samples were suspended in 0·5 ml of a solution containing propidium iodide, (0·05 mg ml⁻¹, Calbiochem). This is a fluorescent nucleic acid dye that counterstains DNA

red against the green fluorescence from the fluorescein staining of the p62^{c-myc}. Thus one control contained only nuclei stained with propidium iodide, the other was stained with propidium iodide and the second antibody (fluorescence control), and the remaining four samples were stained with the varying dilutions of anti p62^{c-myc} plus fluorescent second antibody and propidium iodide.

Fluorescence assay The nuclei were analysed with the Cambridge Medical Research Council custom built flow cytometer.^{8,9} This instrument constrains the nuclei to flow in fluid suspension through a high light collection efficiency flow chamber¹⁰ and to pass single file through the focus of a Coherent Innova 90 argon ion laser (Palo Alto, California, United States of America) tuned to the 488 nm line at a light power of 100 mW. This laser line excites green fluorescence from fluorescein tagged oncoprotein and red fluorescence from propidium iodide stained DNA. The two fluorescence signals from each nucleus, together with forward and 90° light scatter were quantitated by photodetectors. The fluorescence emission was separated by a dichroic beam splitter that reflects light below and transmits above 580 nm. The green and red photomultipliers were additionally guarded by a 515–560 nm band pass and a 630 nm long pass filter, respectively. Even with this degree of optical filtration about 0·01% of the propidium iodide stained DNA signal entered the green photomultiplier, which was an advantage in this assay. The instrument was set up on the control stained by propidium iodide alone, so that the diploid G1 peak was recorded in channel 200 on the DNA (red) axis and at channel 50 on the p62^{c-myc} (green) axis. This enabled the instrument to be set up identically for each run.

Data collection and analysis The data were collected list mode on a fast RP07 disc via a dedicated LSI 11/23

Fig. 3 *C-myc* oncoprotein assay in CIN 3 disease. Left hand panel phosphate buffered saline control plus propidium iodide and DNA staining. DNA is plotted on ordinate as contour map against signal from green detector channel. Middle panel: fluorescence control, fluorescent second antibody only. Right hand panel: p62^{c-myc} fluorescence.

Fig. 4 CIN 3 disease; display identical to that of Fig 3.

and a time sharing PDP 11/40 computer (all from Digital Equipment Corporation, Maynard, Massachusetts, United States of America). After acquisition the data were recalled from disc by our inhouse analysis programs and displayed as contour plots of DNA (ordinate) v p62^{c-myc} (abscissa) after gating on forward and right angle scatter to exclude clumps and debris.

Results

SPECIFICITY CONTROLS

Antibody specificity controls have been described previously.⁴⁻⁶ Briefly, four monoclonal antibodies, which do not recognise p62^{c-myc} or nuclear proteins, gave no signal above background, and specific fluorescence was blocked by preincubation of Myc 1-6E10 with the peptide used as the immunogen.

CERVICAL BRUSH SPECIMENS

Figs. 3-5 show three representative sets of data. All

three samples were obtained and analysed on the same afternoon, and they represent the first three samples run after preliminary sampling and fixation procedures had been investigated. Fixation procedures included wooden spatula and Cyto-Brush biopsy specimen collection, each with formal saline, acetone, acetone and methanol, and methanol fixation. The combination of Cyto-Brush biopsy plus methanol gave the highest cell yield with the best epitope preservation. All sets of data in Figs. 3-5 are directly comparable, and each shows red fluorescence (DNA) on the ordinate v the signal from the green photodetector channel on the abscissa. The left hand, middle, and right hand panels, respectively, show the propidium iodide control, fluorescence control (second antibody only), and p62^{c-myc} (Myc 1-6E10 plus second antibody) sets of data. In each of the left hand panels the contour display is angled away from the Y axis due to breakthrough of the red propidium iodide and DNA signal into the green channel, and the G1, S, and G2M plus components of the DNA histogram are

Fig. 5 Mild dysplasia with inflammatory disease. Note increased p62^{c-myc} signal in right hand panel compared with that of Figs. 3 and 4.

clearly discernible. In the middle panels there is a small increase in the signal on the abscissa due to non-specific trapping of the second antibody; the X axis distribution is slightly right shifted compared with that of the respective propidium iodide controls. A small specific p62^{c-myc} fluorescence signal was observed in the right hand panels of Fig. 3 and 4, but a large signal was observed in the right hand panel of Fig. 5. Conventional cytology corresponding to cells analysed in Figs. 3 and 4 showed cervical intra-epithelial neoplasia (CIN) 3, grade III and IV atypical cells. Similar analysis of cells corresponding to Fig. 5 showed only mild atypia with inflammatory change.

Discussion

We have described cervical sampling using the Cyto-Brush, which is suitable for both conventional cytology and the simultaneous flow cytometric assay for DNA and p62^{c-myc}. The purpose of the study was to determine the optimum procedure suitable for an automated prospective cervical screening program using oncoprotein probes and flow cytometry. The cell yield with the Cyto-Brush was considerably greater than we had expected and would have been sufficient for analysis using several monoclonal probes. This method also has the advantage that the squamocolumnar junction can be biopsied (Fig. 2).

The three samples used in the Figs. were from two patients with CIN 3 disease and one with only mild atypical changes. The first two patients had low p62^{c-myc} values and the third high values. These findings were in keeping with preliminary results (in preparation) obtained from paraffin wax embedded cervical biopsy specimens, using a similar technique.⁵ The relatively higher p62^{c-myc} values in normal cervical epithelium compared with neoplastic epithelium has been a consistent finding. This type of correlation has also been observed with this antibody in testicular teratoma, in which there was a direct correlation between p62^{c-myc} nuclear content and degree of differentiation. Tumours with yolk sac elements exhibited the highest values.⁶

Several automated cervical prescreening procedures have been developed using both flow and microscope based image analysis systems. These include slit-scan nuclear and cytoplasmic ratios,¹² measurement of DNA content,¹³⁻¹⁷ DNA content plus total protein¹⁸ and image analysis assaying DNA and chromatin "texture".^{19,20} These methods are based either on morphology or non-specific biochemical markers (DNA and total protein). This may not always be sufficient to make a reliable distinction, as morphology, DNA content, and chromatin "texture" need not reflect the malignant phenotype. The method described here, using the Cyto-Brush for sample col-

lection and an oncoprotein probe, may help to resolve some of the potential problems associated with analysis developed to date. Our methods are being directed towards biochemical assays in normal and malignant cells that may reflect either qualitative or quantitative differences in specific proteins which are thought to play a part in growth regulation and proliferation control. p62^{c-myc} is one such protein.^{5,21-24}

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Letters to the Editor

Identification of immunoreactive atrial natriuretic peptide in atrial amyloid

Amyloidosis is a disorder characterised by the deposition of an abnormal proteinaceous material in the extracellular tissues.^{1,2} It may occur as a primary disease process or secondary to a variety of conditions characterised by chronic inflammation. It has been described in association with tumours and occurs in hereditary forms. It occurs in a localised or systemic distribution.

Amyloid has characteristic staining reactions, but is best defined by its appearance on electron microscopy where fine non-branching fibrils about 10 nm in diameter can be identified. Despite the similarity of appearance the chemical structure of the fibrils varies with site and disease associ-

ation. In systemic primary and myeloma associated amyloid the fibrils contain protein derived from immunoglobulin light chains. In secondary amyloid the fibrils contain a protein related to the acute phase reactant serum amyloid A. In hereditary or familial amyloidosis and in cerebral amyloid of Alzheimer's disease they contain prealbumin. Amyloid localised to endocrine organs may contain hormone related peptides—for example, immunoreactive calcitonin is found in amyloid associated with medullary carcinoma of the thyroid.³⁻⁵

Amyloid affects the heart as part of systemic amyloidosis or in a localised form as a manifestation of aging.⁶ Two types occur in the aging heart: that affecting both the ventricles and atria and that affecting the atria alone, known as isolated atrial amyloid.⁷ Isolated atrial amyloid has been reported in 78% of patients over the age of 80 years: its origin is unknown.

Recent evidence suggests that the heart is

an endocrine organ, and peptides arising from the atria have been isolated and characterised. They have potent natriuretic, diuretic, and vasodilating actions and may play an important part in the homeostasis of body fluids.^{8,9} In this report we describe the immunohistochemical localisation of human atrial natriuretic peptide to amyloid fibrils in human atrial appendage, indicating that some cardiac amyloid is analogous to that seen in other endocrine organs.

Material and methods

A fresh piece of right atrial appendage removed at coronary bypass surgery was fixed in paraformaldehyde lysine sodium periodate¹⁰ for 24 hours. It was then divided to provide blocks for routine paraffin histology and electron microscopy. Paraffin sections were cut at 4 μ m and stained with haematoxylin and eosin, sulphated alcian blue, and alkaline Congo red for amyloid.¹¹

Fig. 1a Electron micrograph of human atrial appendage showing labelling of amyloid fibrils for human atrial natriuretic peptide. (Immunogold/uranyl acetate lead citrate, 15 nm gold balls.) $\times 56\,000$.

Fig. 1b Electron micrograph of human atrial appendage showing labelling of neurosecretory granules for human atrial natriuretic peptide. (Immunogold/uranyl acetate lead citrate, 15 nm gold balls.) $\times 112\,000$.