Comparison of in situ methods to assess DNA cleavage in apoptotic cells in patients with breast cancer

P N Mainwaring, P A Ellis, S Detre, I E Smith, M Dowsett

Abstract

Background—Apoptosis has a role in many cellular processes including development, normal tissue homeostasis, and malignancy. This aspect of research is relatively new with distinct methods of analysing disparate biochemical and genetic events to measure apoptotic cells. The use of biotinylated nucleotides to identify DNA strand breaks is a commonly reported method of estimating cells numbers undergoing apoptosis; however, investigators report inconsistent results for a variety of reasons.

Aims and method—To compare two in situ techniques of measuring apoptosis: in situ nick translation (ISNT) and TdT mediated dUTP–biotin nick end labelling (TUNEL); and to assess DNA cleavage in 20 paired paraffin wax embedded breast cancer tissues from patients; one group who had received no prior treatment and one group who had received chemohormonal treatment.

Results and conclusions—Apoptotic scores obtained from paraffin wax embedded human breast cancer after using ISNT and TUNEL methods were not significantly different (p = 0.11). A strong correlation between scores obtained from the two techniques was found (r = 0.758, p < 0.0001). Optimisation of both techniques is crucial to ensure maximal assay performance in breast cancer tissue.

(Keywords: apoptosis, in situ nick translation; TUNEL; in situ end labelling; breast cancer)

In vitro and in vivo evidence strongly points to induction of apoptosis as the primary mechanism by which malignant breast cells die in response to systemic treatment. The current, most widely accepted standard for measuring apoptosis in vivo involves light microscopic evaluation using criteria established in the original descriptions of this mode of cell death. Time lapse studies show that induction of apoptosis may take as little as 1–3 hours and apoptotic cells and bodies may appear for only a few minutes to hours before they are phagocytosed. Thus cells that are recognisable apoptotic on morphological criteria are relatively scarce in tissue sections and laboured to count on slides using conventional stains. In addition to cell shrinkage, apoptosis is characterised by chromatin condensation and nuclear fragmentation. Investigations are currently in progress dissecting the order of molecular and biochemical events associated with programmed cell death. A broad spectrum of cell lines grown under different culture conditions have implicated cleavage of double stranded DNA, in some cases by endogenous Ca++/Mg++ endonucleases, into oligonucleosomal fragments, as an event indicating irreversible commitment to apoptosis.

In response to the scarcity of apoptotic cells and difficulties in recognition and counting, two other methods have been described to label DNA breaks in nuclei. These methods have eased detection of apoptosis by light microscopy. One method, the TUNEL assay, is based on incorporating biotinylated deoxyuridine (b-dUTP) at 3'-OH DNA strand breaks using binding with terminal deoxynucleotidyl transferase (TdT). The other method, known as in situ nick translation (ISNT) or in situ end labelling (ISEL), uses the Klenow fragment of Escherichia coli DNA polymerase I for incorporating the b-dUTP. The former method targets 3' recessed, 5' recessed, and blunt end DNA fragments, while the latter favours recessed 3' ends.

There has been a major increase in the number of studies of apoptosis, and the application of these two techniques. The TUNEL assay has proved popular because of the availability of a kit form. In one study measuring induction of apoptosis by flow cytometry the kinetics of biotinylated dUTP incorporation was faster and the distinction of cells with DNA breaks was more pronounced using TUNEL; in the other TUNEL was reported to demonstrate apoptotic cells in breast cancer tissues while ISNT did not. In contrast, our experience in uncontrolled studies indicated that the two techniques may be similar in identifying cells undergoing apoptosis. We therefore conducted a controlled comparative study of these two approaches for the detection of cells committed to apoptosis to aid us and others in choice of technique.

Material and methods

Formalin fixed, paraffin wax embedded breast cancer tissues from two patient groups were analysed. One group had surgical excision of their disease without prior chemotherapy. The other underwent primary or neoadjuvant chemohormonal treatment, comprising methotrexate, mitoxantrone, and tamoxifen (MMT), before resection of residual disease as part of a recently reported randomised study. Two sets of 20 breast carcinomas, one set before and one after three months of...
combination chemohormonal treatment, were randomly selected from our histopathology stores. Pairs of 3µm sections were cut, deparaffinised, and taken through progressively decreasing concentrations of industrial methylated spirit to double distilled water (DDW). Endogenous peroxidase activity was inactivated with 1% hydrogen peroxide in phosphate buffered saline (PBS) pH7.4, for 10 minutes. Nuclei of tissue sections were stripped of proteins by incubation with 0.5% pepsin, pH2, (Sigma Chemical Co, Poole, Dorset, UK) for 30 minutes at 37°C. The sections were washed five times in DDW to remove all traces of pepsin.

Each section undergoing the TUNEL protocol was incubated for five minutes in Tris buffer, pH 7.6, and then for one hour at 37°C in 100 µl of reaction mixture consisting of 15 units TdT FPLC pure (Pharmacia, Windsor, Berkshire, UK), 0.5 nmol biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany), 5 mM cobalt chloride, 0.2 M sodium cacodylate, 25 mM Tris HCl, pH 6.6, and 0.25 mg/ml bovine serum albumin (BSA), fraction V, dissolved in DDW.

After extensive washing in DDW all the sections were incubated for 30 minutes at room temperature in 1/400 dilution of horseradish peroxidase conjugated to streptavidin (Dako, High Wycombe, Bucks, UK) in PBS supplemented with 1% BSA and 0.5% Tween 20. Colour was developed for 10 minutes using 0.05% diaminobenzidine plus 0.07% imidazole plus 0.1% hydrogen peroxide, and further intensified in 0.5% copper sulphate with 0.9% sodium chloride for one minute. The sections were counterstained in Mayer’s haematoxylin, dehydrated, cleared in xylene, and mounted in DPX.

The slides were separately scored by two observers using a double headed eyepiece attachment to a Nikon microscope. With the aid of a graticule within an eyepiece, three thousand malignant cells (±1%) were counted at ×400 magnification, taking approximately 30 minutes per slide. In slides where there was disagreement in the scores exceeding 15% the slide was rescoring by both observers and a mean of the two scores for each observer recorded (six slides being rescoring). Stained apoptotic cells were recorded and cells displaying classic apoptotic morphology but not staining (always <30% of the stained apoptotic cells/section, and generally <15%; mean 5.2%) were also incorporated in the apoptotic index. Non-staining apoptotic cells were recognised in the midst of cells with normal morphology by having either condensed, irregular nuclei frequently with a crescent shaped appearance, or fragmented nuclei within cells showing cytoplasmic withdrawal, resulting in a halo-like effect. Areas with extensive necrosis were avoided. Otherwise, different fields of view were assessed at convenience.

Statistical analyses assumed a skewed distribution and were therefore non-parametric. Correlations were analysed using Spearman rank correlation and paired comparisons using the Wilcoxon signed-rank test. All analyses were performed using the Statview v4.5 statistical program (Abacus Concepts, Berkeley, California, USA).

**Results**

All tumours were histologically infiltrating ductal carcinomas. Of the 40 patients’ tissue sections selected for study, two patients from the MMT treated group were not suitable for analysis, one set because of inadequate residual breast cancer and another because extensive ductal carcinoma in situ predominated over...
Table 1: Apoptotic scores of breast cancer tissues

<table>
<thead>
<tr>
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<th>ISNT</th>
<th>TUNEL</th>
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<tr>
<td>Untreated group (median) (range)</td>
<td>0.41% (0.04-2.44%)</td>
<td>0.49% (0.07-2.64%)</td>
</tr>
<tr>
<td>MMT group (median) (range)</td>
<td>0.39% (0.03-2.03%)</td>
<td>0.47% (0.06-2.03%)</td>
</tr>
<tr>
<td>Overall</td>
<td>0.41% (0.03-2.44%)</td>
<td>0.48% (0.06-2.64%)</td>
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There was a difference in background staining between the two techniques however this did not affect the ability to interpret and score the malignant cells (fig 1). The median apoptotic scores and ranges are shown in table 1. There was no significant difference in apoptotic scores between sections scored with the ISNT technique and those scored with the TUNEL technique overall (p = 0.11), or in the adjuvant and neoadjuvant groups considered separately (p = 0.20 and p = 0.33, respectively). A strong correlation between scores was demonstrated between the two techniques overall (r = 0.758, p < 0.0001) (fig 2). Comparisons between the groups treated with adjuvant compared to primary/neoadjuvant therapy were not made.

Discussion

The rate of growth of a tumour is a balance between the rate of proliferation and the rate of cell death. We have demonstrated that this balance is altered in postmenopausal women with breast cancer treated with tamoxifen as well as anceis alterm in postmenopausal women with cell death. We have demonstrated that this balance between the rate of proliferation and the rate of cell death is a balance.

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Measurement of apoptosis

eck, and mononuclear cell cultures from patients with myelodysplastic syndromes, but not in breast cancer tissues whether neoplastic, normal or metastatic, in HL-60 cultured cells treated with etoposide or serum-starved A549 lung carcinoma cells.\textsuperscript{10, 14} Mean apoptotic indices in double labelled myelodysplastic cultured cells, ISNT and TUNEL, were comparable to those in single labelled cells in each case. In contrast, serum starved A549 lung carcinoma cells demonstrated apoptosis detectable only by TUNEL and not by ISNT in single and double labelling experiments. These authors have hypothesised that specific fragmentation patterns could be a result of activation of different endonucleases that could be tissue specific and may be differentially activated by different chemotherapeutic agents.\textsuperscript{10, 14}

These differences would then be reflected in the staining patterns of apoptotic cells because of the different specificities for DNA cleavage by the enzymes involved, potentially leading to major differences in the estimation of apoptosis according to the method employed, the disease/tissue studied, and the treatment context.

We chose to assess treated and untreated tumours so that the possibility could be evaluated that apoptosis occurring during chemotherapy might yield apoptotic cells that rendered them systematically more sensitive to staining by one or the other method. Our finding of good correlation between the two techniques in both untreated and treated breast carcinomas, with the treatment involving cytotoxic and hormonal agents, suggests that any such differences are unlikely to have a significant impact on estimations made in breast carcinomas. It should be noted that we would not necessarily expect the apoptotic score to be higher than pretreatment values after three months' treatment as the resected disease may be chemoresistant by that time.

In preparation for our study we, like others,\textsuperscript{15} found that optimising protein cross linking digestion in paraffin wax embedded tissues clearly influenced our efforts to minimise background staining while maximising that of apoptotic cells. To circumvent problems associated with proteolytic treatment of paraffin wax embedded tissues Panchalingham and colleagues reported pretreatment by pressure cooking before assessment of apoptotic cells using ISNT and TUNEL.\textsuperscript{16} Using this method they obtained similar results between sections stained with ISNT or TUNEL in tissues from patients with Burkitt's lymphoma.\textsuperscript{16} Two studies on human brain tissue have reported reduction of apoptotic index counts with prolonged formalin fixation and reversal of this loss with microwave pretreatment.\textsuperscript{17, 18} A recent paper has reported that using higher magnifications of tissue sections, stained with haematoxylin and eosin, increased correlation coefficients of

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