In situ end-labelling, light microscopic assessment and ultrastructure of apoptosis in lung carcinoma

E F Gaffney, A J O'Neill, M J Staunton

Abstract

Aims—To compare in situ end-labelling (ISEL) of apoptosis in lung carcinoma with quantitative and semiquantitative light microscopic assessment and ultrastructural observations.

Methods—ISEL of apoptosis was evaluated in 42 lung carcinomas (24 squamous cell carcinomas, 12 adenocarcinomas and six small cell carcinomas). Results were correlated semiquantitatively with the extent of apoptosis in haematoxylin and eosin stained sections, with apoptotic indices and with ultrastructural observations (nine cases).

Results—In each tumour type the extent of apoptosis identified by ISEL correlated with that observed on light and electron microscopy. Tumour cells undergoing apoptosis showed either uniform nuclear staining with a surrounding "halo" or peripheral nuclear membrane staining. The latter pattern was more prominent in small cell carcinoma and correlated ultrastructurally with early apoptosis. A variable proportion of apoptotic cells and apoptotic bodies were unlabelled. Necrotic tumour cells were weakly stained but were distinguishable from apoptotic cells.

Conclusions—ISEL, if used in conjunction with standard methods for investigating apoptosis, is a useful adjunct to the investigation of apoptosis in human tumour tissue.

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Apoptosis is the genetically mediated process whereby individual cells are deleted from normal and diseased tissues.¹ The methods most widely used to identify apoptosis are light and electron microscopy,²⁻⁶ flow cytometry,⁷ and agarose gel electrophoresis.⁸ However, because of the small size and typically scattered distribution of apoptotic cells and apoptotic bodies in spontaneous human tumours high magnification objectives are needed for studying apoptosis on routine light microscopy. Recently, the in situ end-labelling (ISEL) technique has been used to identify apoptosis in paraffin wax sections.^{9,10} The principle of ISEL is that the DNA of apoptotic cells becomes fragmented into regular nucleosome sized units, the overhanging 3'-OH ends of which can be identified by the immunohistochemical

detection of enzymatically incorporated digoxigenin labelled triphosphates such as dUTP.⁹⁻¹¹

Although ISEL has been used to identify apoptosis in human tumours,¹²⁻¹⁴ there is very little data correlating ISEL findings with standard light and electron microscopic evaluation of apoptosis in tumours. We therefore examined the use of ISEL in the assessment of apoptosis in lung carcinomas, correlating the findings with semiquantitative and quantitative determination of apoptosis on routine light microscopy and with ultrastructural observations.

Methods

Formalin fixed, paraffin wax embedded sections from 42 lung carcinomas (24 squamous cell carcinomas, 12 adenocarcinomas and six small cell undifferentiated (oat cell) carcinomas) were studied. All, except three (mediastinal or hilar lymph nodes with metastatic small cell carcinoma), were pneumonectomy or lobectomy specimens.

ISEL was performed using an apoptosis detection kit (Oncor, Gaithersburg, Maryland, USA; supplied by Alpha Laboratories, Eastleigh, Hampshire, UK) according to the manufacturer's instructions with the following exceptions: (1) endogenous peroxidase was blocked using 2% hydrogen peroxide in methanol for 10 minutes; (2) proteinase K (40 µg/ml) was applied to sections in a humidified chamber at 37°C for 15 minutes; (3) 35-40 µl of working strength TdT enzyme was added per slide; (4) one drop of anti-digoxigenin peroxidase was added per slide; and (5) sections were stained with 3,3'-diaminobenzidine, for seven minutes, and methyl green (five dips). Sections of testis, containing meiosis induced, double stranded DNA breaks, were used as controls. Apoptosis identified by ISEL was assessed semiquantitatively on a scale of 0-5 (0 = no apoptosis, 1 = rare apoptotic cells/bodies, 2 = focal, 3 = diffuse, not extensive, 4 = multifocal, 5 = very extensive).

Apoptotic indices were determined in haematoxylin and eosin stained sections as previously described, avoiding zones of necrosis.⁶ In addition, apoptosis was assessed semiquantitatively using a similar scale (0-5) to that used for the assessment of ISEL.

Nine cases (four squamous cell carcinomas, two adenocarcinomas, three small cell carcinomas) were examined by electron microscopy, specifically to identify and characterise

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Figure 1 Apoptosis in small cell undifferentiated carcinoma. The nuclear chromatin of apoptotic tumour cells is irregularly condensed or peripheral. Toluidene blue, $\times 400$.

Figure 2 In situ end-labelling of apoptosis in squamous cell carcinoma ($\times 400$).

Figure 3 In situ end-labelling of apoptosis in small cell undifferentiated carcinoma. Most apoptotic tumour cells in this field show irregular nuclear membrane staining (see fig 1), but a minority show staining of shrunken nodular chromatin ($\times 400$).

the ultrastructure of apoptosis. Six specimens were fixed initially in 2% glutaraldehyde. The other three specimens had been fixed initially in 10% neutral buffered formalin and subsequently placed in 2% glutaraldehyde. All tissue was post-osmicated and processed for electron microscopy. From resin blocks, 0.5 μ m semithin sections were cut and stained with toluidene blue for light microscopy. Ultrathin sections from selected blocks were stained with uranyl acetate and lead citrate, and examined in a Hitachi 7100 electron microscope.

Results

In all three tumour types apoptotic cells and apoptotic bodies were scattered among viable tumour cells and were often also concentrated in the non-viable rim of necrotic zones. Small cell undifferentiated carcinoma had more apoptosis than either squamous cell carcinoma or adenocarcinoma; apoptotic cells, with peripherally beaded or shrunken nodular condensed chromatin, and apoptotic bodies occurred in clusters among viable tumour cells, with no associated inflammatory response. The cytological detail of cells undergoing apoptosis was more clearly seen in semithin toluidene blue sections (fig 1).

On ISEL, squamous cell carcinomas and adenocarcinomas showed moderate to strong staining of the shrunken nuclear chromatin in apoptotic cells. These cells usually had a perinuclear "halo" and often had cell membrane condensation (fig 2). Some apoptotic cells and apoptotic bodies showed enhanced staining of the nuclear membrane with reduced or absent staining of the remainder of the nucleus. Occasional, apparently normal tumour cell nuclei were also labelled. A variable proportion of morphologically identifiable apoptotic cells and apoptotic bodies did not stain.

In small cell carcinomas apoptotic tumour cells predominantly showed an irregular nuclear membrane ISEL pattern (fig 3). This ISEL pattern correlated closely with the morphology of apoptosis seen in toluidene blue (fig 1) and ultrathin sections (see later). Many apoptotic cells and apoptotic bodies were not labelled.

On ISEL, there was also weak cytoplasmic staining of tumour cells that had phagocytosed apoptotic tumour cells or lymphocytes. Macrophages showed cytoplasmic staining and necrotic cells showed homogeneous weak staining that differed from that seen in apoptotic cells (fig 4). In necrotic zones apoptotic cells were seen in the non-viable rim and occasionally mixed with central necrotic tumour cells or polymorphs. Very scattered labelled apoptotic cells were identified within bronchiolar epithelium and in alveolar spaces in areas of bronchopneumonia distal to the tumour.

Apoptotic indices ranged from 0.1 to 5.4% (mean 1.3%) for adenocarcinoma, 0.04 to 7.9% (mean 2.3%) for squamous cell carcinoma and 5.4 to 14.0% (mean 10.9%) for small cell carcinoma. Semiquantitative assessment of apoptosis reflected apoptotic indices for each tumour type—for example, most adenocarcinomas had a score of 1 and five of

Figure 4 Necrotic tumour cells stain faintly in contrast to an apoptotic tumour cell (top left) in squamous cell carcinoma ($\times 400$).

Figure 5 Ultrastructure of apoptosis in small cell carcinoma. The apoptotic tumour cell shows irregular, dense, maze-like subnucleolemmal condensation of nuclear chromatin (original magnification $\times 14\,000$).

six small cell carcinomas had a score of 5. Semiquantitative apoptosis scores of 2–4 (each based on an entire section) often did not correspond exactly to the apoptotic indices, which were based on the assessment of 10 high power fields only. However, in all tumours a semiquantitative score of 2–4 reflected an intermediate extent of apoptosis, as determined by the apoptotic indices. Semiquantitative ISEL scores were comparable (identical or with a score difference of 1) to the semiquantitative assessment of apoptosis in 27 of 34 tumours. Finally, the extent of apoptosis identified in toluidene blue and ultrathin sections correlated with light microscopic and ISEL determinations of apoptosis for the three small cell carcinomas only, because of sampling limitations in the other tumours, which had less apoptosis.

Small cell carcinomas showed the spectrum of ultrastructural cellular alterations associated with apoptosis. In two cases tumour cells showed predominantly early apoptotic features with irregular peripheral subnucleolemmal or maze-like transnuclear condensation of granular osmiphilic chromatin (fig 5). The third case, which had less apoptosis, contained more uniformly osmiphilic apoptotic nuclei, interpreted as representing a later stage of apoptosis (fig 6). Compared with small cell carcinoma there was minimal apoptosis in squamous cell carcinoma or adenocarcinoma. In both adenocarcinoma and squamous cell carcinoma occasional phagocytosed apoptotic bodies and tumour cells exhibiting late apoptotic changes (fig 7) were seen. One adenocarcinoma had rare tumour cells in early apoptosis.

Discussion

The proportion of tumour cells stained by ISEL and their distribution within the principal lung carcinoma types correlated well with the assessment of apoptosis on light and electron microscopy. The different ISEL patterns observed emphasise the morphological diversity of apoptosis. A variable number of apoptotic cells in each tumour were not labelled by this method, and we cannot exclude the possibility that DNA breaks in certain non-apoptotic tumour cells were also identified by ISEL, as suggested previously.¹⁰ However, we consider that the great majority of tumour cells identified by ISEL were unquestionably apoptotic, given the close correlation of ISEL results with assessment on light and electron microscopy. On ISEL, apoptotic tumour cells were distinguishable from weakly labelled necrotic cells. Finally, we suggest that cytoplasmic staining observed in macrophages possibly represents staining of DNA that has undergone further enzymatic degradation.

It has been suggested¹⁵ that peripheral nuclear membrane staining, as illustrated herein, represents early apoptosis.^{16,17} Our combined ISEL and ultrastructural findings support this. This was the predominant ISEL pattern seen in small cell carcinoma, although it was also observed to a lesser extent in squamous cell carcinoma and adenocarcinoma. This implies that the majority of apoptotic cells identified by ISEL in small cell carcinoma are in the early stages of apoptosis, clusters of tumour cells having simultaneously undergone apoptosis, as in morphogenesis or in tumour regression. Indeed, a peripheral nuclear staining pattern was also illustrated by Fukasawa *et al* in regressing myofibromatosis.¹² Extensive peripheral nuclear labelling may therefore be indicative of particularly widespread apoptosis, but this remains to be examined in greater detail. Other possible explanations are that in small cell carcinoma a different apoptosis inducing stimulus is associated with different apoptosis morphology, that adjacent tumour cells and macrophages effect more rapid clearance of late stage apoptotic cells from the tumour tissue, and that the later stages of

Figure 6 A shrunken apoptotic tumour cell, with dense nuclear chromatin, is engulfed by an adjacent tumour cell in small cell undifferentiated carcinoma (original magnification $\times 12\,000$).

Figure 7 Late stage apoptosis in poorly differentiated squamous cell carcinoma (original magnification $\times 12\,000$).

apoptosis are not labelled for technical reasons, such as conformational changes in DNA that render end-labelling sites inaccessible.¹⁸

Classic morphological apoptosis is associated with internucleosomal DNA fragmentation, often preceded by transient breaks in single or double stranded DNA.^{19–21} The large DNA fragments formed initially are undetectable by standard agarose gel electrophoresis,^{22–24} but stepwise degradation of subsequent 10–40 kilobase fragments into oligonucleosomes produces the distinctive DNA ladder pattern. However, apoptosis has been reported to occur in the absence of DNA fragmentation, with no ladder pattern,^{22,25} or with a delayed ladder pattern.²⁶ A DNA ladder pattern has also been

reported without morphological evidence of apoptosis.²⁷ DNA breaks alone are not sufficient evidence of apoptosis because they may occur in response to various endogenous or exogenous mutagens.^{28,29} It is unclear how DNA breaks in susceptible cells escape normal DNA repair mechanisms and undergo secondary DNA fragmentation.²¹ DNA fragmentation alone does not cause structural collapse and condensation of chromatin, and in certain systems the two processes may be triggered by separate pathways.³⁰ It remains to be determined whether DNA fragmentation is a consistent feature of apoptosis in spontaneous human tumours.

In conclusion, we consider that ISEL would facilitate a better understanding of the process of apoptosis in tumours. Not only are there several possible induction mechanisms for apoptosis but there may also be differences in the manner of DNA fragmentation among different tumour types, as reported in certain experimental systems. Like most other techniques used in the assessment of apoptosis, ISEL is not completely sensitive or specific. For this reason, and because it is as yet a relatively untried method, results of ISEL should be interpreted in conjunction with apoptosis assessment by routine light microscopy, electron microscopy or another standard technique.³¹

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