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A COMPARISON OF THE ANTI-HISTONE AND APOP-TAG TECHNIQUE FOR DEMONSTRATING APOPTOSIS WITH OPTION FOR SILVER ENHANCEMENT

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A novel immunocytochemical method is presented for the qualitative detection of DNA fragmentation in apoptosis. Anti-histone antibody is employed to localize exposed nucleosomal histones (H1, H2a, H2b, H3 and H4) rather than tagging the cut ends of fragmenting DNA as in conventional technique. The method was tested on squamous cell carcinoma of the larynx routinely fixed in formaldehyde and embedded in paraffin wax and compared with results obtained employing Apop-Tag kit (Oncor).

KEYWORDS: apoptosis; anti-histone; Apop-Tag; silver enhancement; laryngeal carcinoma

INTRODUCTION

DNA fragmentation is widely regarded as an important, though not exclusive characteristic of apoptosis (Wyllie, 1981; Bellamy *et al*., 1995), and in-situ labelling of the cut-ends of endonuclease released DNA is an established technique (Wijsman *et al*., 1993). Investigators have reported variable results using such techniques (Bowen *et al*., 1998) and a useful alternative based on immunocytochemical localization of released nucleosomal histone is presented here.

The method is based on selective development of an ELISA technique presented by Trauth *et al*. (1995) (Boehringer Mannhein Biochemica). The method is applicable for in-situ histological localisation of apoptosis and has been applied in a preliminary study of programmed cell death in the honey bee *Apis mellifera* (Gregorc and Bowen, 1997), although few methodological details were given in this latter paper.

The incidence of apoptosis in tumour tissue is well established (Sarraf and Bowen, 1988) and in this paper we compare the in-situ localization of apoptosis obtained with Apop-Tag kit (Oncor) with that of the modified anti-histone kit (Boehringer Mannheim) in a squamous cell carcinoma of the larynx. In this instance, routine paraffin embedded technique was selected since a method based on such an approach would also be available for many retrospective and archival pathological studies.

MATERIALS AND METHODS

Tumour tissue was obtained from a laryngectomy specimen from a middle-aged male. Histological examination showed this to be moderately differentiated squamous cell carcinoma with no perineural or perivascular spread (clinical stage T4 N0 M0). The squamous cell carcinoma was fixed in 10% formalin for 24 h. Dehydrated in 70%, 90% and 100% alcohol for 1.5 h at each concentration. The sample was cleared in Histoclear for 2 h and wax-impregnated by placing the sample in 50:50 Histoclear:wax for 1 h in the oven (60°C) followed by three changes in pure paraffin for 45 min, 1 h and 3 h at 60°C. The tissue was then embedded in fresh wax and the block was cut using a Bright microtome into sections 5 μm thick, layed in a waterbath (45°C) and picked up on subbed slides and dried on a slide drier.

The following immunocytochemical techniques were performed on the paraffin sections:

(1) Anti-histone Antibody technique with and without silver/gold intensification.

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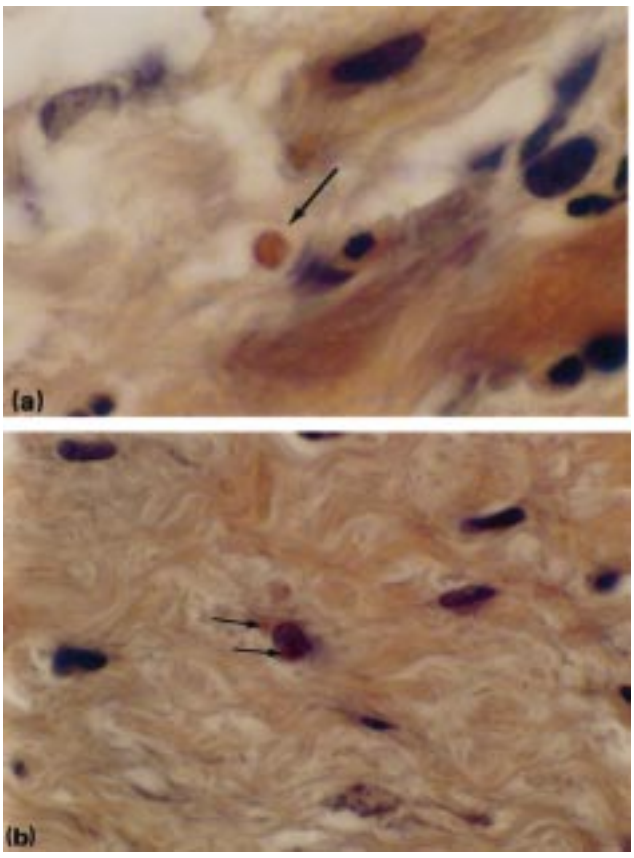


Fig. 1. (a) An apoptotic cell treated with the anti-histone technique without the silver/gold intensification. Note brown colouration of an apoptotic body (arrow) Mag. (× 1320). (b) Note discrete brown apoptotic bodies (arrows) next to a normal cell, in material treated with the anti histone method without silver intensification. Much of the tumour background appears to be highly fibrous. Mag. × 1320, reproduced at 55%.

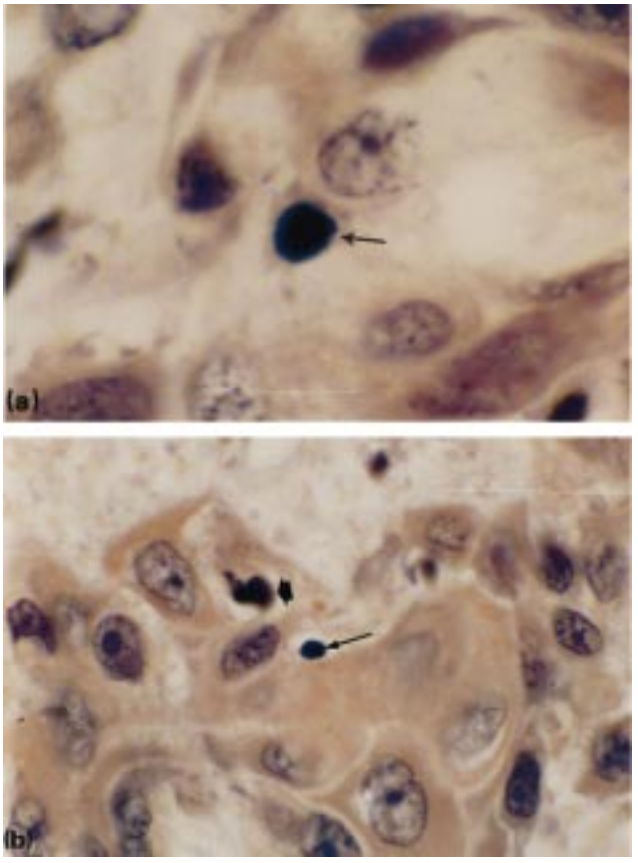


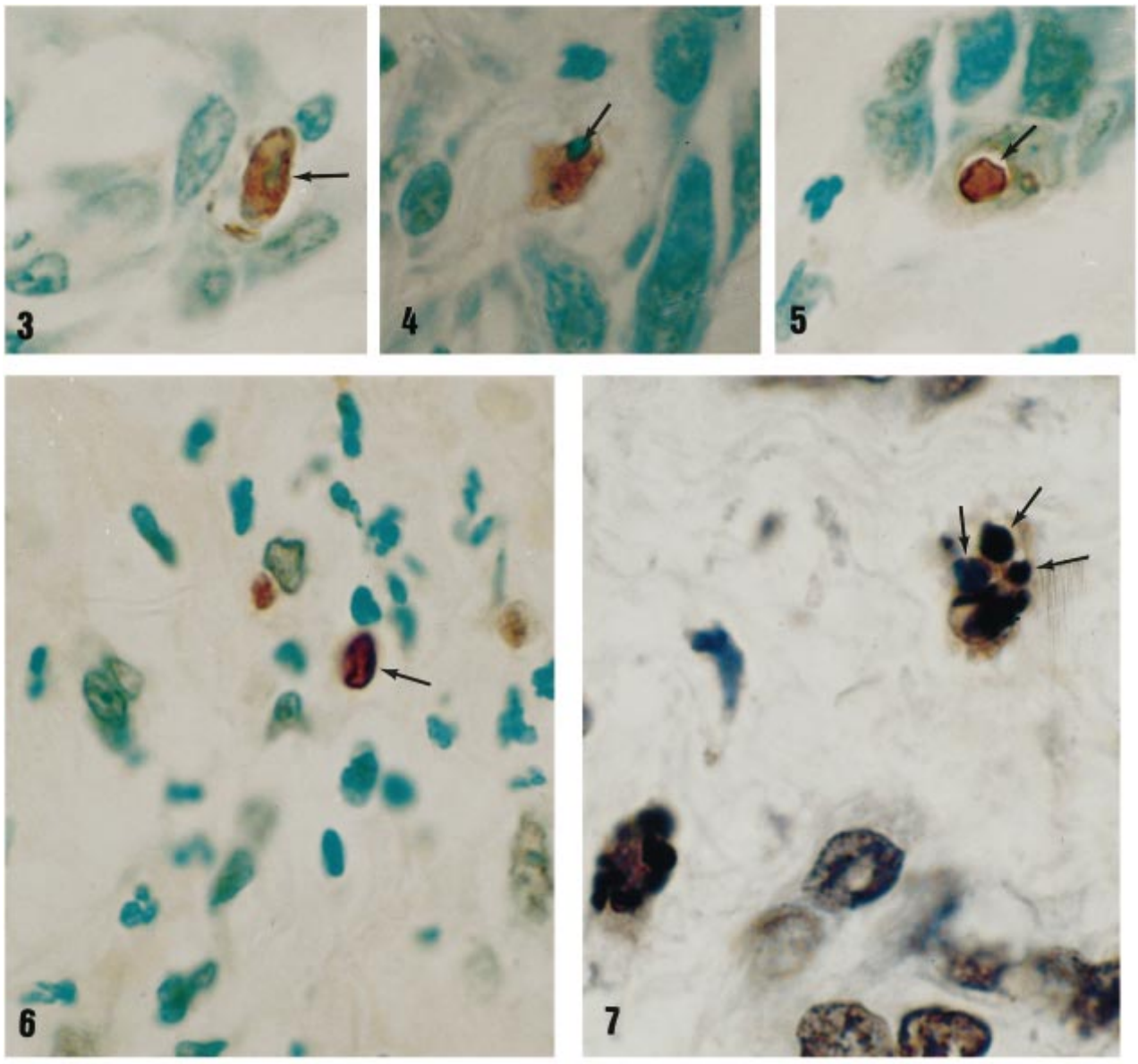
Fig. 2. (a) An apoptotic cell treated with the anti-histone technique and with the silver/gold intensification (arrow). Mag. × 1320. (b) The anti-histone method with silver intensification. Note the occurrence of one small shrunken apoptotic cell (small arrow) and also a florid apoptotic profile (large arrow) near to normal looking tumour cells. Mag. × 1320, reproduced at 55%.

(2) Apop-Tag technique with and without silver/gold intensification.

The anti-histone antibody technique

Without intensification. The paraffin sections were dewaxed in three changes of xylene and 3 changes of alcohol, 5 min each change. Endogenous peroxidase was blocked with a mixture of 75.5 ml methanol and 1.3 ml hydrogen peroxidase for 30 min at room temperature, followed by washing in tap water for 5 min. The sections were placed in 0.01 M phosphate buffer saline (PBS) pH 7.1, then removed individually from PBS and blotted, without allowing the sections to dry and placed in a moist chamber. The sections were covered with 200 µl of anti-histone (conc. 1.5 µg/ml Tris buffer + 1% BSA pH 8.2) and left at 4°C overnight, and then washed in three changes of PBS, 1 min each wash. The sections were once again removed from PBS, blotted, and then placed in a moist chamber. 200 µl of mouse immunoglobulin conjugated with peroxidase was applied on each section and left for 1 h at room temperature, then washed in 3 changes of PBS, 1 min each. The sections were placed in diaminobenzidine dihydrochloride (DAB)/hydrogen peroxidase (H_2O_2) for 5 min (0.05 g DAB in 100 ml PBS then 4 drops in H_2O_2 was added), and then rinsed in PBS. (For the silver/gold intensification the sections were kept in PBS overnight at room temperature.)

The sections were washed in running tap water for 5 min, then placed in 1% aqueous acetic acid for 30 s. Counterstaining was done with Mayer's haematoxylin for 1 min, and washed well in running tap water for 5 min, then blued in Scott's tap water for 1 min. Dehydrated in three changes of alcohol, then cleared in three changes in xylene, 5 min each change and mounted in DPX.



Figs 3–7. Fig. 3. The Apop-tag technique demonstrating an early apoptotic cell counterstained with methyl-green (arrow). Mag. (× 1320). **Fig. 4.** The blebbing of an apoptotic cell with the Apop-Tag technique. The methyl-green staining of the nucleolus is clear (arrow). Mag. (× 1320). **Fig. 5.** The shrinkage of an apoptotic cell and the appearance of the nuclear gap with the apop-tag technique. Mag. (× 1320). **Fig. 6**. A late apoptotic cell where the chromatin margination is clear (arrow). Mag. (× 1320). **Fig. 7**. Numerous apoptotic bodies (arrows) with the silver/gold intensification of the Apop-Tag technique. Mag. (× 1320).

Silver/gold intensification. This was performed according to Rollinson and Butler (1997). The sections were taken out of PBS and incubated in 10% thioglycolic acid for 2 h in room temperature to suppress non-specific binding. Sections were washed in 2% sodium acetate (four washes, 30 min each) and placed in reaction solution for 8 min. The solution was made up as follows:

Solution A: 50% sodium carbonate.

Solution B: 1 g of ammonium nitrate dissolved in 250 ml distilled water, then 1 g of silver nitrate was added. 5 g of tungstosilicic acid was dissolved in 250 ml distilled water and added slowly to solution (B). 400 µl of formalin was added to 100 ml of solution B. This solution was added to the 100 ml solution A.

Washed in 2% sodium acetate (2 washes, 5 min each wash), placed in 0.05% gold chloride (2

washes, 5 min each wash), washed in 2% sodium acetate (2 washes, 5 min each wash), washed in 3% sodium thiosulfate (2 washes, 5 min each wash), washed in 2% sodium acetate (2 washes, 5 min each wash), and washed in PBS.

Then the same counterstaining protocols as for without intensification were followed.

Apop-Tag technique (Wijsman *et al.*, 1993)

Without silver/gold intensification. The Apop-Tag *In Situ* Apoptosis Detection Kit (Oncor) was used to detect apoptotic cells by direct immunoperoxidase reporting of digoxigenin-labelled genomic DNA in paraffin-embedded tissue as follows.

The tissue sections were deparaffinized by three changes of xylene and three changes of alcohol (5 min each change), then washed in PBS for 5 min. Proteinase K (20 µ/ml) was applied directly on the section for 15 min at room temperature to digest the protein in the sections; after this, sections were washed in four changes of distilled water for 2 min. Endogenous peroxidase was quenched in 2.0% hydrogen peroxidase in PBS for 5 min at room temperature. Rinsed with PBS, for 5 min each time. Excess liquid was tapped off and blotted around each section and immediately 2 drops of 1 × Equilibration Buffer (provided in the kit) was applied on the specimen. Plastic coverslips (provided in the kit) were used and the sections were incubated for 10–15 s at room temperature (slide may be left in Equilibration Buffer for up to 30 min).

The plastic coverslips were removed and the excess liquid tapped off and blotted around each section and immediately 54 µl of Working Strength TdT Enzyme was applied on each section and covered with a plastic coverslip and incubated in a humidified chamber at 37°C for 1 h. (The TdT enzyme provided in the kit is supplied concentrated in a stabilized buffer to preserve activity.) Therefore, 1 drop of TdT Enzyme was diluted with 2 drops of reaction buffer. This reagent must be kept in ice once prepared.

The plastic coverslips were removed and the specimens were put in a Coplin jar containing Working Strength Stop/Wash Buffer agitating for 15 s, and incubated for 10 min at room temperature. This buffer was prepared by adding 1 ml of Stop/Wash Buffer (provided in the kit) to 34 ml distilled water and pre-warmed to 37°C. Specimens were then washed in three changes of PBS for 5 min each.

Two drops of Anti-Digoxigenin-Peroxidase was applied to the slides and covered with a plastic coverslip and incubated in a humidified chamber for 30 min at room temperature. The sections were then washed in 3 changes of PBS for 5 min each.

Hydrogen peroxide (0.02) was added to 0.05 g DAB in 100 ml PBS and immediately the slides were covered with this solution for 5 min at room temperature, washed in 3 changes of distilled water, for 1 min each wash. Then washed in distilled water for 5 min.

Specimens were counterstained with 0.5% methyl green in 0.1 M sodium acetate, pH 4.0 for 10 min at room temperature. Washed in three changes of distilled water, dipping the slide 10 times each in the first and second washes, followed by 30 s without agitation in the third wash. Washed in 3 changes of 100% alcohol, dipping the slide 10 times each in the first and second washes, followed by 30 s without agitation in the third wash. Rinsed in 3 changes of xylene for 2 min each wash, then mounted in DPX.

Apop-Tag technique (with silver/gold intensification)

After the DAB peroxidase reaction the sections were kept in PBS overnight to be silver enhanced the next day.

The silver/gold intensification is the same as mentioned earlier for the anti-histone technique.

RESULTS

The anti-histone technique

The anti-histone antibody technique without silver/gold intensification gave positive results. The brown colour in Figure 1(a) reflects the endonuclease fragmentation of DNA which is associated with apoptosis and localizes the site of antibody attachment. During cell death endonuclease is activated, and cleaves the internucleosomal linker regions of DNA, normally the region of DNA which is tightly complexed to the histone is protected. The cleavage of the inter-nucleosomal linker regions of the DNA, expose the histones which can be detected by monoclonal antibodies raised in mice against these histones.

Figure 1(b) presents a larger field than that shown in Figure 1(a). The tumour is seen to be moderately differentiated with large amounts of fibrous collagen between the cells. Dark brown reaction product indicative of endonuclease activity is in this case seen in small individual apoptotic bodies next to a tumour cell.

The silver/gold intensification of the anti-histone antibody technique attempts to improve the immunoperoxidase DAB method. This technique results in a higher contrast that the use of DAB alone. Figure 2 demonstrates an apoptotic body with this intensification. Figure 2(b) shows a wider field than that depicted in Figure 2(a) and demonstrates one compact shrunken apoptotic cell near to a larger florid apoptotic cell surrounded by tumour cells.

Unenhanced, positive apoptotic nuclei appear brown, but contrast can be improved with silver intensification (compare Figs 1 and 2). In both cases some care is needed to avoid excessive counterstaining otherwise all the nuclei end up looking black.

The Apop-Tag technique

The Apop-Tag technique demonstrates DNA fragmentation and the exposure of the cut end regions of DNA. The brown colour that appears in an early apoptotic cell Figure 3 indicates DNA fragmentation as reported by an anti-digoxigenin DAB localized peroxidase. Figure 4 demonstrates the blebbing of an apoptotic cell. The methyl green staining of the nucleolus of this cell is very clear.

At a later stage, Figure 5 shows the shrinkage of an apoptotic cell and the appearance of a gap which separates the apoptotic body from the surrounding neighbours.

Figure 6 demonstrates a late apoptotic body where the chromatin margination is clear. The silver/gold intensification of the Apop-Tag treated sections was successful, the dark apoptotic bodies appear clearly in Figure 7.

DISCUSSION

The development of reliable in-situ localization of apoptosis related DNA fragmentation is an important area of tumour research (Bowen and Bowen, 1990; Bowen, 1998). In this paper an alternative to DNA labelling is presented, in that the immunocytochemical target is exposed nucleosomal histone groups. DNA based techniques such as the Apop-Tag method could give rise to artefacts based on non-endonuclease fragmentation of DNA, such as can occur during necrosis. The anti-histone method specifically focusses on the exposure and release of nucleosomal histones and thus more specifically ties in with endonuclease fragmentation of the linker regions of DNA.

The pattern of localization produced by both methodologies is similar and is based on DAB reaction with reporter peroxidase. Both methods result in a brown product observable in apoptotic nuclei and apoptotic bodies. The contrast produced by the anti-histone technique is not as great as that produced with the anti-DNA Apop-Tag technique and thus, silver enhancement (Rollinson and Butler, 1997) of the immunocytochemical products of both techniques have been explored. Such an enhancement leads to a black deposit of high contrast.

The results produced by both methods were comparable but the anti-histone method is more specific, shorter, less expensive and easier to carry out. It is also easily modified for use at electron microscope level (Al-Hazzaa and Bowen, 1998). Incidentally, it was noted that the methyl green counterstain appeared to give a better nuclear differentiation although haematoxylin counterstain was used routinely for the anti-histone method in this case. The advantages of employing methyl green staining in cell death studies have recently been outlined by Moffitt (1994).

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