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Simultaneous detection of DNA fragmentation (apoptosis), cell proliferation (MIB-1), and phenotype markers in routinely processed tissue sections

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Abstract In situ DNA fragmentation assays have proved to be particularly useful in the detection of apoptosis in routinely processed, paraffin-embedded tissue sections. In the present study, a triple-antigen labelling technique was performed to demonstrate DNA fragmentation (apoptosis), cell proliferation (MIB-1), and phenotypic markers in the same tissue section. The in situ apoptosis assay was conducted with the TUNEL method developed by a avidin-biotin alkaline phosphatase complex (ABcomplex/AP). The proliferation-associated MIB-1 antigen was demonstrated in the second staining sequence by the avidin-biotin peroxidase method (ABC). The phenotypic markers chromogranin A or prostate-specific antigen (PSA) were visualized by the alkaline phosphatase anti-alkaline phosphatase method (APAAP) in the third staining sequence. The feasibility of this triple-labelling technique was tested in formalin-fixed, paraffin-embedded tissue of prostatic adenocarcinomas from 8 patients with recurrent, hormone-refractory disease. Although these tumours revealed marked neuroendocrine differentiation, cell proliferation and apoptosis were detected exclusively in non-endocrine (chromogranin A-negative) tumour cells that expressed PSA variably. The triple-labelling protocol described here allows the phenotypic characterization of proliferating and apoptotic cell populations in the same tissue section. It may be useful in studies of tissue kinetics in physiological and pathological processes.

Key words Apoptosis · Proliferation · Phenotypic markers

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Introduction

Normal growth in human tissue is regulated by a delicate balance between cell proliferation and cell death. Disruption of the molecular mechanisms that regulate these two processes results in abnormal growth in benign and malignant conditions. Apoptosis or programmed cell death is defined by a single-cell phenomenon characterized by DNA fragmentation and nuclear shrinkage [8]. Novel in situ DNA fragmentation assays have proved particularly useful in the detection of apopotosis in routinely processed, paraffin-embedded tissue [1, 6, 10]. Assessment of cell proliferation has become possible since the introduction of the Ki-67 equivalent MIB-1 antibody, which identifies cycling cells in formalin fixed, and archival tissue [4]. In addition to cell proliferation and apoptosis, a growing number of cytoplasmic biomarkers, including differentiation antigens and oncogene and tumour suppressor gene products, are currently available for basic tissue research. More information on the regulatory impact of biomarkers on normal and abnormal tissue growth can be provided by studies investigating their relative expression in relation to cell proliferation and apoptosis. Such investigations, however, require the simultaneous demonstration of different markers in the same tissue section.

In the present study, we describe a triple-labelling technique that allows simultaneous detection of DNA fragmentation (apoptosis), cell proliferation (MIB-1) and phenotypic markers in human tissue. The feasibility of this triple-label procedure was tested in prostatic adenocarcinoma showing marked neuroendocrine (NE) differentiation. This particular phenotype has attracted increasing attention in contemporary prostate cancer research [5]. Recent clinico-pathological studies have shown that NE differentiation predicts tumour progression after radical prostatectomy or radiation therapy [7, 9]. Preliminary results obtained in the current study indicate that endocrine tumour cells assessed by the pan-endocrine marker chromogranin A (ChrA) lack both proliferative and apoptotic activity.

Materials and methods

The material submitted for study had been selected from a larger series of prostatic adenocarcinomas previously screened for NE differentiation using the pan-endocrine marker ChrA. Formalin-fixed, paraffin-embedded tissue sections were obtained from eight patients who underwent palliative transurethral resection for recurrent prostate cancer after androgen ablation therapy. Tumour samples investigated showed at least ten low-power fields with numerous ChrA-positive tumour cells.

The terminal transferase-mediated biotinylated 16-desoxy-uridine-tri-phosphate (dUTP) nick end labelling (TUNEL) assay was performed as described previously with minor modifications on paraffin-embedded, formalin-fixed tissue sections [2, 5, 8]. Briefly, the sections were dewaxed in xylene, rehydrated through graded ethanol at room temperature, and pretreated with proteinase K (Boehringer Mannheim, Germany) in phosphate-buffered saline (10–20 µg/ml) at 37°C in a humidified chamber for 10–30 min. After washing twice in distilled water and rinsing in Tris-buffered saline (TBS; 0.15 M NaCl; 0.05 M Tris-HCl; pH 7,6) for 5 min, the sections were incubated for 60 min at 37°C with a reaction mixture containing the reaction buffer (200 mM potassium cacodylate; 25 mM Tris-HCl; BSA 0.25 mg/ml; pH 6,6), 1.5 mM cobald cloride, 0,05nM biotin 16-dUTP, and 0.5 U/µl terminale transferase (Boehringer Mannheim, Germany). The reaction was terminated by rinsing twice in TBS for 10 min. The sections were covered with 2% (w/v) bovine serum albumin (BSA; Sigma, Deisenhofen, Germany) for 15 min at room temperature and incubated with avidin–biotin-conjugated alkaline phosphatase (ABC-AP; Dako, Hamburg, Germany) at a concentration of 1:100 in TBS at room temperature for 30 min. The staining was achieved with 5-bromo-4-chloro-3-indoxyl phosphate/nitro blue tetrazolium (BCIP/NBT alkaline phosphatase Kit II, Vector Laboratories, Burlingame, Calif.) according to the manufacturers instructions, leaving a blue/purple end-product. In all experiments, sections of lymph node were stained as positive controls. For negative controls the terminal transferase was omitted from the reaction mixture.

For MIB-1 immunolocalization in the second staining sequence, the sections were treated with 0,6% H_2O_2 for 30 min to block endogenous peroxidase, and were heated in 0.01 M citrate buffer (pH 6) in a microwave oven for 2 min at 700 W and 4 min at 300 W. The slides were allowed cool to room temperature, rinsed in TBS and incubated in rabbit normal serum (Dako) for 30 min. The MIB-1 monoclonal antibody (Dianova, Hamburg, Germany) was applied at a dilution of 1:20 in TBS overnight at 4°C in a humidified chamber. A biotinylated monoclonal rabbit anti-mouse (Dako) was used as secondary antibody (dilution 1:200 in TBS; 30 min). The specimens were then stained using the avidin–biotin complex horseradish peroxidase technique (Dako) according to the manufacturers instructions, using diaminobenzidine (Sigma) as chromagen.

After the slides had been blocked with normal rabbit serum (Dako) for 30 min, the monoclonal mouse antibodies against chromogranin A (Novocastra, Newcastle upon Tyne, UK) or PSA (Dianova) were applied in a dilution of 1:20 in TBS for 1 h at 37°C. A monoclonal, nonbiotinylated rabbit anti-mouse (Dako) was used as secondary antibody in a dilution of 1:50 in TBS for 30 min. The alkaline phosphatase anti-alkaline phosphatase complex (APAAP complex; Immunotech, Hamburg, Germany) was applied in a dilution of 1:50 in TBS for 30 min. To intensify the immunereaction the secondary antibody and the APAAP complex were applied twice in a dilution of 1:100 in TBS. The staining was completed by the New Fuchsin Substrate Pack (Biogenex, San Ramon, Calif.) leaving a red end-product. The slides were faintly counterstained with haematoxylin (Merck, Darmstadt, Germany). For negative controls the primary antibodies were omitted from the second and third staining sequence.

Results

In routinely processed, paraffin-embedded and archival tissue, reliable detection of apoptotic cells by the TUNEL assay requires careful evaluation of the staining results. The standard protocol described above may yield inconsistent findings with regard to the expected number of apoptotic cells. The duration of proteinase K pretreatment and the enzyme concentration were found to be the most critical points in the DNA fragmentation assay. Underdigestion yielded false-negative results, whereas overdigestion produced false-positive results and increased background staining. For each individual case, the optimal pretreatment conditions had to be determined using

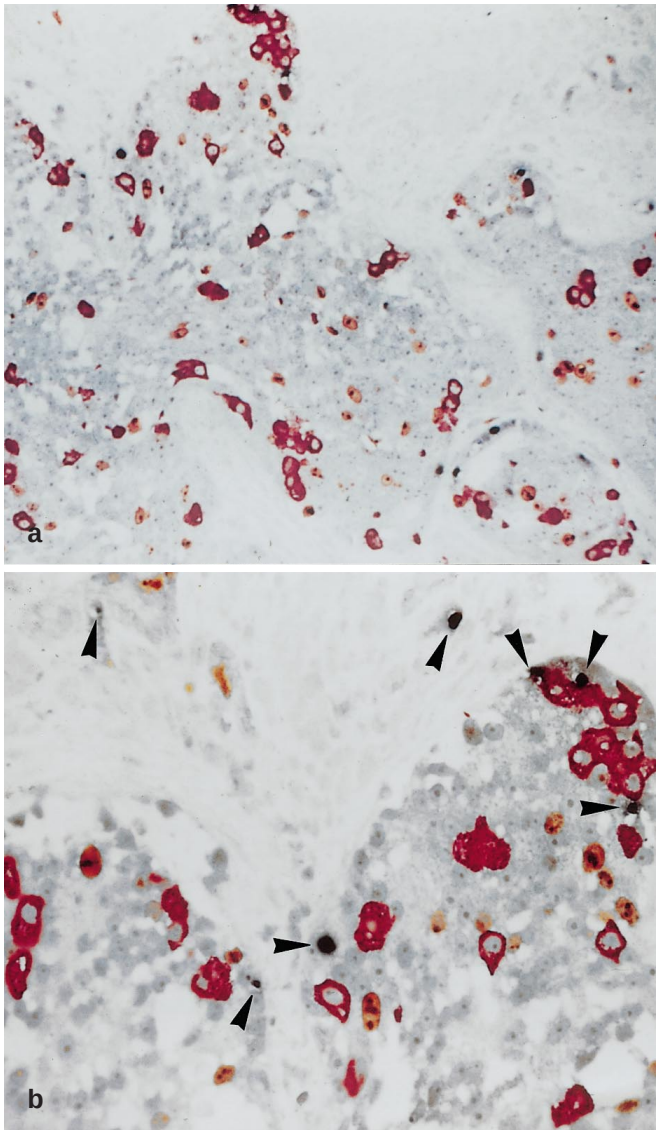

Fig. 1a, b Prostatic adenocarcinoma with marked neuroendocrine differentiation. Simultaneous detection of DNA fragmentation (*dark blue, arrowheads*), MIB-1 (*brown*), and chromogranin A (*red*). Chromogranin A positive tumour cells lack both the proliferation-associated MIB-1 antigen and detectable DNA strand breaks (apoptosis). Original magnifications **a** ×100, **b** ×400

suitable positive and negative controls. Prostatic stromal cells, which are known for their very low apoptotic activity, and luminal spaces of apoptotic cells, which are normally present in pre-existing duct-acinar structures, served as internal controls. Slides with optimal staining results were submitted to microwave irradiation, which did not interfere with the stability of the coloured endproduct of the apoptosis assay. The subsequent immunohistochemical double-stainings were made possible by use of different enzyme systems and coloured endproducts. Results of this triple-labelling protocol allowed reliable identification of DNA fragmentation (apoptosis), cycling (MIB-1 positive) cells and cytoplasmic markers (ChrA, PSA) in the same tissue section (Fig. 1). Omission of the in situ apoptosis assay did not interfere with MIB-1, ChrA or PSA immunolocalization obtained in single-staining experiments. In our series of prostatic adenocarcinoma with marked NE differentiation, we failed to demonstrate unequivocal colocalization of DNA fragmentation or MIB-1 immunoreactivity in ChrA-labelled (endocrine) tumour cells. Conversely, MIB-1 or DNA fragmentation was detected exclusively in ChrA-negative tumour cells with variable expression of PSA (Fig. 1).

Discussion

Results of the current study show that simultaneous detection of DNA fragmentation (apoptosis), cycling (MIB-1 positive) cells and phenotypic markers is feasable in the same tissue section. The most critical step in the triple-labelling technique reported here is the in situ apoptosis assay. Suitable positive and negative controls are important for reliable interpretation of the staining results [1, 10]. The subsequent immunohistochemical double-staining requires microwave-based antigen retrieval, which may seriously interfere with the stability of the coloured end-product of the apoptosis assay performed in the first staining sequence. The BCIP/NBT complex used as chromogen in the in situ apoptosis assay resists microwave pretreatment, making subsequent immunohistochemical analysis possible with appropriate antigen retrieval conditions. More importantly, the in situ apoptosis assay appears not to interfere with the immunolicalization of antigens detected in the second and third staining sequences In our experiments, omission of the in situ apoptosis assay did not alter the staining results obtained with MIB-1, PSA or ChrA antibodies in single stainings.

The feasibility of this triple-labelling protocol in formalin-fixed, routinely processed tissue was demonstrated in prostatic adenocarcinoma, which is a particularly good example of phenotypic heterogeneity in human neoplasms. Common prostatic adenocarcinoma is composed mainly of exocrine (PSA-positive) tumour cells. The second most important phenotype shows NE differentiation detected by pan-endocrine markers such as ChrA [5]. NE differentiation frequently occurs in common prostatic adenocarcinoma and has potential prognostic implications, as shown by recent clinico-pathological studies [7, 9]. Results of the current study suggest that proliferation activity and apoptosis occur exclusively in exocrine (ChrA-negative) cell populations with variable expression of PSA. This confirms previous data on the proliferation status of endocrine–paracrine tumour cells in prostate cancer [3]. This particular phenotype occurs exclusively in the G_0-phase of the cell cycle and is lost when tumour cells re-enter the cycle [2, 3]. The observation that endocrine tumour cells do not undergo apoptosis may have important implications for the biology of this phenotype and warrants further investigation.

The triple-antigen-labelling technique described in the present study allows simultaneous detection of DNA fragmentation, cell proliferation, and phenotypic markers in the same tissue section. This rapid, sensitive and reproducible method is suitable for evaluation of growth fractions and of the phenotype of proliferating and apoptotic cell populations in human tissues.

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