IMMUNOCYTOCHEMICAL DETECTION AND MAPPING OF A CYTOKERATIN 18 NEO-EPITOPE EXPOSED DURING EARLY APOPTOSIS

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SUMMARY

A neo-epitope in cytokeratin 18 (CK18) that becomes available at an early caspase cleavage event during apoptosis and is not detectable in vital epithelial cells is characterized. The monoclonal antibody M30, specific for this site, can be utilized specifically to recognize apoptotic cells, which show cytoplasmic cytokeratin filaments and aggregates after immunohistochemistry with M30, while viable and necrotic cells are negative. The number of cells recognized by the antibody increases after induction of apoptosis in exponentially growing epithelial cell lines and immunoreactivity is independent of the phosphorylation state of the cytokeratins. The generation of the M30 neo-epitope occurs early in the apoptotic cascade, before annexin V reactivity or positive DNA nick end labelling. In a flow cytometric assay, the majority of the M30-positive cells appear in the ‘apoptotic’ subG1 peak. Tests with synthetic peptides define positions 387–396 of CK18, with a liberated C-terminus at the caspase cleavage site DALD-S, as the ten-residue epitope of M30. This epitope starts at the end of coil 2 of the predicted CK18 structure, at a probable hinge region, compatible with the sensitivity to proteolytic cleavage. The definition of a specific caspase cleavage site in CK18 as a neo-epitope can be used for quantification of apoptotic epithelial cells with immunocytochemical techniques and is applicable to both fresh and formalin-fixed material. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—apoptosis assay; caspase cleavage site; cytokeratin; monoclonal antibody; neo-epitope

INTRODUCTION

Apoptosis is a biochemically and morphologically distinct form of programmed cell death, initiated by specific signals that activate specific caspases.1 As a result, the cell is eliminated by an intrinsic suicide programme, resulting in DNA fragmentation, nuclear condensation, cytoskeletal reorganization, plasma membrane blebbing, and loss of cell adhesion.2 Methods widely used to identify apoptotic cells are light and electron microscopy, flow cytometry,3 agarose gel DNA electrophoresis, in situ nick-end labelling (ISEL), TdT-mediated dUTP nick-end labelling (TUNEL),4 and recent methods based on the detection of phosphatidylserine (PS) in the outer plasma membrane using annexin V.5 In studies aimed at the production of monoclonal antibodies directed to cytokeratin 18 (CK18), we noticed that one antibody, named M30, recognized epithelial cells which from their morphological pattern appeared to be apoptotic. That observation led to the identification of a caspase cleavage site in CK18 and to the characterization of a neo-epitope that is exposed only during apoptosis. In the course of this study, it became evident that a positive staining reaction with M30 can be regarded as an early visible event in the apoptotic cascade.

MATERIALS AND METHODS

M30 monoclonal antibody

The monoclonal antibody M30 was produced by immunization of Balb/c mice with two purified CK18 fragments from cell culture medium6 from the human colonic carcinoma cell line WiDr CCL218 and subsequent spleen cell hybridization with mouse myeloma cells (P3x63-Ag 8.653, kindly supplied by Professor G. Köhler, The Max Planck Institute of Immunology, Freiburg, Germany).

Immunohistochemistry

Epithelial WiDr CCL218 cells, grown on coverslips for 3 days and fixed in methanol at −20°C overnight and in 96 per cent ethanol, were incubated with M30 at 0.5 µg IgG/ml for 30 min at room temperature, followed by peroxidase (PO)-labelled anti-mouse Ig (DAKO A/S, Glostrup, Denmark).

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The human squamous non-small cell lung cancer cell line MR65, a kind gift of Dr Gropp (Philips Universität Klinik, Marburg, Germany), was grown on coverslips for 1 day and fixed for 5 min in methanol at −20°C. The cells were incubated with M30 for 1 h at room temperature, followed by fluorescein isothiocyanate (FITC)-conjugated F(ab′)2 fragments of rabbit anti-mouse IgG (F313; DAKO A/S, diluted 1:10). The cells were counterstained with 5 μg/ml propidium iodide (PI; Calbiochem, La Jolla, CA, U.S.A.) and RNAse (1 mg/ml SERVA, Heidelberg, Germany) for 7 min. The cells were finally embedded in glycerol/DABCO/PI [9:1 glycerol, 1 part 0.2 M Tris-HCl (pH 8.0), 2 per cent DABCO, 5 μg/ml PI, 0.02 per cent NaN3].

Five-micrometre thick sections from formalin-fixed, paraffin-embedded colon carcinomas were incubated, after antigen retrieval by microwave heating for 15 min at 700 W in citrate buffer (0.2 M, pH 6.0), with M30 at 1 μg IgG/ml for 1 h at room temperature. Biotinylated rabbit anti-mouse Ig (DAKO) was followed by PO-labelled streptavidin (DAKO). The PO labels were visualized with aminoethylcarbazole (AEC; Sigma, St Louis, MO, U.S.A.) in 0.002 per cent H2O2, resulting in a red precipitate. Nuclear counterstaining was done with haematoxylin.

To see whether or not M30 immunoreactivity depended on hyperphosphorylation of cytokeratins, phorbol ester-treated cells (see below) were double-stained with RCK106 (IgG1 subtype), a monoclonal antibody against CK18,7 and M30 (IgG2b subtype). After fixation in methanol at −20°C for 5 min, the cells were incubated with both primary antibodies for 1 h at room temperature. Antibody binding was visualized using FITC-conjugated rabbit anti-mouse IgG1 and TxRed-conjugated rabbit anti-mouse IgG2b (Southern Biotechnology Associates, Birmingham, AL, U.S.A.).

To compare the reaction pattern of M30 with that of other apoptosis markers, such as the specific exposure of DNA strand-breaks was done by the incorporation of DIG-11-UTP (Boehringer Mannheim, Almere, The Netherlands) and detection with FITC-conjugated Fab fragments of sheep anti-digoxigenin (Boehringer Mannheim) in a TUNEL assay as described before.4

DNA was counterstained using DAPI (5 μg/ml; Sigma). Confocal images were recorded using a Bio-Rad MR600 confocal scanning laser microscope equipped with an air-cooled argon/krypton mixed gas laser. DAPI fluorescence was recorded in a non-confocal mode.

Induction of cytokeratin aggregation

In order to evaluate M30 reactivity under non-apoptotic conditions, cytokeratin aggregation was induced in MR65 cells by means of the phorbol-ester method of Töll et al.8 Briefly, phorbol 12-myristate 13-acetate was added to conditioned culture medium at a final concentration of 0.15 μM for 1 h at 37°C. The cells were then washed twice with phosphate-buffered saline (PBS) at 0°C and incubated with PBS for 1 h at 0°C. Alternatively, induction of cytokeratin aggregation was induced by treatment of cells with a hypotonic buffer (90 mM NaCl, 3 mM KCl, and 10 mM Tris/HCl, pH 7.3) for 1 h at 0°C.

Flow cytometry

M30 reactivity was compared with the results of the annexin V and TUNEL assays in trivariate M30/annexin V/DNA and M30/TUNEL/DNA flow cytometric (FCM) analyses.

For FCM analysis of M30 monoclonal antibody binding, DNA content, and TUNEL reactivity, MR65 cells were incubated to undergo apoptosis using etoposide. Cells were harvested by trypsinization and fixed for 5 min in methanol at −20°C. Visualization of DNA strand-breaks using the TUNEL assay and M30 monoclonal antibody binding was performed on suspended cells as described above. Cellular DNA was counterstained using PI (5 μg/ml; Calbiochem, La Jolla, CA, U.S.A.) in PBS (pH 7.4) containing 0.1 mg/ml RNAse (SERVA). Analyses were performed using a FACSort flow cytometer equipped with an air-cooled argon ion laser (Becton Dickinson, Sunnyvale, U.S.A.).

Western blotting

MR65 cells treated with etoposide for 4.5 or 7 h were harvested by scraping and subjected to SDS/polyacrylamide gel electrophoresis followed by western blotting. Antibodies against poly(ADP-ribose) polymerase (anti-PARP, BioMol, Plymouth Meeting, PA, U.S.A.) and CK18 (RCK106) were applied in parallel with M30. Monoclonal antibody binding was visualized using peroxidase-conjugated rabbit anti-mouse Ig (DAKO), with subsequent detection using enhanced chemiluminescence (ECL kit, Amersham, Essex, U.K.).

M30 epitope mapping

In order to characterize the M30 epitope, the antibody was tested against a number of synthetic oligopeptides, synthesized using the multipin approach utilizing polyethylene supports derivatized with an acid handle.9 Peptides representing both the N- and C-terminal truncation series of the optimally reacting sequence EDFNLGDALD and other sequences were synthesized. They were capped with biotin using the tetrapeptide linker sequence -SGSG- or -SGSB- and attached to streptavidin-coated plates in the enzyme-linked immunosorbent assay. Reactivity between the monoclonal antibody M30 and the chemically synthesized
Fig. 1—Formalin-fixed, paraffin-embedded colonic carcinoma (a), cultured colonic carcinoma cells (WiDR CCL218) (b) and cultured non-small cell lung cancer cells (MR65) (c) exposed to the M30 monoclonal antibody.

(a) Cells with apoptotic morphology such as chromatin condensation (arrow 1), nuclear fragmentation (arrow 2), and detachment of cytoplasm from the environment (arrows 1 and 2) showed cytoplasmic staining for M30. Progressively degraded cells (arrow 3) are negative for M30, as are the tumour cells with intact morphology. Some cells show clear granular cytoplasmic staining (arrow 4).

(b) M30-reactive WiDR cell showing a filamentous staining pattern.

(c) M30-reactive MR65 cell showing the distinct nuclear apoptotic morphology, i.e. chromatin condensation and fragmentation. Note that cells with intact nuclear morphology, including the metaphase cell, are devoid of M30 staining.

(d) Triple labelling of CK18 (green), M30 (red), and DNA (blue) of MR65 cells after phorbol-ester treatment. Note that the cytokeratin aggregates are devoid of M30 immunoreactivity.

Fig. 2—(a, b) Triple staining of MR65 cells with the M30 monoclonal antibody (red), DNA (blue), and the annexin V (a) or TUNEL (b) method (green) after apoptosis induction with etoposide. Cells were analysed by confocal scanning laser microscopy and are represented as linear projections of stacks of confocal images. (c, d) Flow cytometric analysis of triple-labelled MR65 cells. Cytograms of TUNEL reactivity (x-axis) and M30 binding (y-axis) at 2–7.5 h (c) and 5–7.5 h (d) after induction of apoptosis with etoposide oligopeptides was investigated in the following manner. Peptides were dissolved at 10 mg/ml in 1:1 acetonitrile–water and diluted with 10 mM PBS, 0.1 per cent Tween 20 to a concentration of 0.01 mg/ml. The ELISA was carried out in the wells of a streptavidin-coated NUNC Maxisorb plate (GIBCO/BRL, Täby, Sweden). After blocking of non-specific absorption, 100 μl aliquots of PO-labelled M30 were added to each well and the plate was incubated for 1 h at 22°C with shaking. After washing, substrate was added to each well and readings were made at 405/492 nm with a Titertek Multiscan instrument (Titertek Instr. Corp., Huntsville, AL, U.S.A.).

RESULTS

When the mouse monoclonal antibody M30 was applied to formalin-fixed, paraffin-embedded colonic adenocarcinoma, this tissue showed a variety of immunostaining reactions (Fig. 1a). Cells with apoptotic morphology, showing chromatin condensation (arrow 1), nuclear fragmentation (arrow 2), and detachment of cytoplasm from the environment (arrows 1 and 2), showed cytoplasmic staining for M30. Progressively degraded cells (arrow 3) were negative for M30, as were the tumour cells with intact morphology. In addition, some cells showed granular cytoplasmic staining (arrow 4).

In WiDr CCL218 cell cultures, a few cells without apparent apoptotic nuclear features, but apparently...
beginning to detach from the surface of the glass slides showed filamentous cytoplasmic staining with M30 (Fig. 1b).

Taken together with the fact that M30 was raised against CK18 fragments and that it shows a filamentous staining pattern next to a granular pattern in several other types of epithelial cells, this indicated to us that the antibody recognizes an epitope on CK18 intermediate filaments.

In exponentially growing non-small-cell lung carcinoma MR65 cells, a small fraction of the cells (±5 per cent) showed a bright, cytoplasmic, granular-like immunofluorescence reactivity pattern with M30, together with features characteristic of apoptotic nuclei, i.e. chromatin condensation and nuclear disintegration (Fig. 1c). Double labelling of the same cell with M30 and RCK106, directed against another epitope of the CK18 antibody, revealed a complete overlap of the immunoreactivity pattern of both antibodies only in apoptotic cells. However, all mitotic cells, in which spontaneous aggregation of cytokeratin filaments is sometimes observed with the RCK106 antibody, were devoid of immunoreactivity with the M30 monoclonal antibody (Fig. 1c). Even after induction of cytokeratin aggregation by phorbol-ester-induced hyperphosphorylation of cytokeratins, aggregated structures remained negative for M30, as shown after double labelling of cells with RCK106 and M30 (Fig. 1d).

A large fraction of the apoptotic cells containing M30 positivity showed membrane affinity for annexin V (Fig. 2a). In the majority of these cells, M30 immunoreactivity was seen in aggregated cytokeratin structures, but in a small fraction a filamentous pattern was seen. A few annexin V-negative cells with a filamentous M30 staining pattern were also found. The nuclei of the latter cells showed no or only minor apoptotic features, while other MR65 cells which showed membranous staining for annexin V, but no staining for M30, exhibited complete nuclear disintegration in almost all cases.

Similarly, M30 immunoreactivity preceded TUNEL reactivity (Fig. 2b). The small fraction of cells showing a filamentous staining pattern with M30 were TUNEL-negative, while the majority of the cells with an aggregated M30 immunoreactivity pattern were TUNEL-positive. Again a fraction of cells were M30-negative and TUNEL-positive, and these cells exhibited complete nuclear disintegration in almost all cases.

In the multivariate flow cytometric analyses of MR65 cells, the fraction of M30-positive cells increased from 2 to 24 per cent within 6 h after induction of apoptosis with etoposide. With time, the fraction of annexin V-reactive cells and of TUNEL-positive cells also increased (Figs 2c and 2d). Positivity for any of the three parameters was accompanied by a decrease in DNA content, resulting in a hypodiploid cell population. M30 reactivity preceded that of the annexin V and the TUNEL assay. Upon extended exposure to etoposide, M30 reactivity decreased, while annexin V binding and TUNEL assay reactivity still remained.

The results obtained by immunocytochemistry were confirmed by western blotting (Fig. 3). Cell lysates of exponentially growing cells were negative for M30, while in cell lysates from apoptotic cultures, two M30-reactive protein bands of approximately 40 and 24 kD were immunostained. The appearance of M30-reactive fragments paralleled PARP cleavage and the appearance of an additional 30 kD cytokeratin fragment detected with RCK106 (Fig. 3a).

When late apoptotic cells (i.e. detached cells) were separated from early apoptotic cells (i.e. cells firmly attached to the culture flask), it was shown that the 40 kD fragment was preferentially found in the early apoptotic fraction, while the 24 kD fragment was mainly detected in the late apoptotic cells (Fig. 3b).

To map the M30 epitope in CK18, the molecule was screened for caspase cleavage sites, using several consensuss motifs including the DALD-X recognition signal. The latter was found to be unique to CK18 and is thus not detected in other cytokeratins known in the protein data banks. Chemical synthesis of peptides corresponding to the relevant cytokeratin segment and to two other potential caspase-cleavage motifs of CK18 revealed that only the CK18-unique segment was indeed
M30-reactive. Further tests, using an array of synthetic peptides corresponding to elongated and truncated segments of the M30-reactive CK18 segment, established that the maximal reactivity was limited to a ten-residue segment ending with the DALD structure (Fig. 3c). Furthermore, molecular modelling using the program ICM11 showed that the most reactive peptide segments could form π helical conformations. This segment is known to occur at the transition of coil 2 to the C-terminal domain of CK18.12

DISCUSSION

Our results establish that early in apoptosis, CK18 is cleaved by caspases, resulting in exposure of an epitope that only then becomes detectable with M30. Combined cytochemical and morphological data link the M30 reactivity to early apoptotic events. Although initially observed with WiDr cells and described here for MR65 cells, the early expression of M30 reactivity in the apoptotic cascade takes place in all cells of epithelial origin that were examined but are not described here in detail, such as epithelia of the gastrointestinal tract, liver, female genital tract, urinary tract, and respiratory tract. Several other cell lines of epithelial origin, including those derived from a mammary carcinoma (MCF7), a cervical carcinoma (HeLa), and a urinary bladder carcinoma (T24), showed staining patterns identical to those described for MR65 (unpublished observations).

CK18 has a widespread distribution and is largely co-expressed with CK5 in practically all simple, non-stratified, ductal and pseudostratified epithelia, while these two cytokeratins are also the first ones to appear in embryonic development. CK18 has been reported to be produced in small amounts also in fibroblasts and other non-epithelial cells.13 Recently, CK filaments have been observed to aggregate rapidly in apoptotic cells14,15 and immunoreactivity with several CK antibodies finally disappears completely.16 This aggregation is mediated by hyperphosphorylation of the cytokeratins.17 Apart from hyperphosphorylation, CK18 is cleaved by a caspase, liberating a neo-epitope which is specifically recognized by the M30 monoclonal antibody. We found that this specific proteolytic cleavage is an event taking place before disruption of membrane asymmetry and DNA strand-breaks occur. From the results of western blotting, it can be suggested that soon after the triggering of the apoptotic cascade, a novel peptide C-terminus is proteolytically liberated on CK18 by cleavage of the peptide bond 396–397. Both the modelling and the findings, it can be suggested that soon after the triggering of the apoptotic cascade, a novel peptide C-terminus is proteolytically liberated on CK18 by cleavage of the peptide bond 396–397. Both the modelling and the

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