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Research Article

An immunohistochemical study of the clearance of apoptotic cellular fragments

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Abstract. We investigated the distribution and fate of apoptotic bodies during human development and in the adult, using an antibody (M30) that recognizes a neo-epitope formed early in the apoptotic cascade by caspase cleavage of cytokeratin 18. In the fetus, we found extensive accumulation of M30-positive, non-phagocytosed fragments in the red pulp of the spleen, subcutaneous and submucosal vessels, the interstitium of the lung, and the glomerular mesangium of the kidneys. In the liver, M30-immunoreactive fragments were found inside macrophages in the sinusoids. The number of these fragments and the intensity of the immunostaining increased with the gestational age of the fetus. In the adult, M30-positive fragments were barely detectable in normal tissues. However, many pathological situations, including both chronic degenerative processes and metastatic cancer, were associated with accumulation of M30-positive fragments in the red pulp of the spleen. In the liver and kidney, no fragments could be detected. Remarkably, 13 of the 16 patients with metastasized cancer showed pronounced accumulation of M30-positive fragments containing hematoxylin-reactive material in the red pulp of the spleen. In the non-cancerous cases, such DNA-containing fragments were only seen in 9 of 94 cases. The results show that when apoptotic activity is high, as during development in the fetus or during metastasis and other pathological processes in the adult, the phagocytic clearance of apoptotic bodies can be overloaded. These apoptotic fragments then accumulate in the spleen. The visual detection of apoptotic fragments is concluded to reflect increased cell turnover.

Key words. Apoptosis; immunohistochemistry; caspase-cleaved keratin; human development; macrophage; spleen; liver.

Normal development of multicellular eukaryotic organisms requires an ordered process of cell proliferation and apoptosis. Apoptosis is an important mechanism not only for maintaining a balance between tissue growth and breakdown but also for the development and differentiation of organs. It plays a major morphogenetic role in the formation of limbs and interdigital clefts [1, 2], the palate [3, 4], and the heart [5], and in the involution of phylogenetic vestiges [6]. Apoptosis first affects scattered individual cells and, once initiated, proceeds rapidly. The characteristic morphological feature of apoptosis is cell shrinkage, chromatin condensation, pycnosis of the nucleus, and nuclear disintegration. The nucleus may disintegrate into fragments (karyorrhexis) [7]. The cell frequently develops cytoplasmic processes that contain condensed nuclear fragments [8]. When these fragments separate from the cells, apoptotic bodies

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are formed which are phagocytosed by monocytes, macrophages, epithelial cells, vascular endothelium, or tumor cells [9].

The average proportion of apoptotic cells that can be seen in a hematoxylin-eosin(H&E)-stained section of normal tissue is generally low, due to the rapid degradation process. We have previously characterized an antibody (M30), recognising a cytokeratin 18 (CK18) neo-epitope that is expressed during the first steps of caspase cleavage in apoptosis [10]. Since this antibody works on formalin-fixed, paraffin-embedded tissues, archival paraffin blocks can be used to monitor apoptotic progress.

In the present study, an immunohistochemical M30 assay was used to investigate the fate of apoptotic epithelial cells during human development and in adulthood. The study focuses on the liver and spleen. The expression of M30-positive activity was compared with staining for CK18, and with the TUNEL assay. A monoclonal antibody directed against the CD68 epitope was used as a macrophage marker.

Materials and methods

Tissue collection and preparation

Tissue samples were collected from 21 fetuses (from spontaneous abortions and still births) ranging in gestational age from 5 to 35 weeks. The samples were fixed in neutral buffered formalin for at least 24 h and subjected to paraffin embedding for routine histological examination with H&E. Similarly, from 110 consecutive autopsies of adult persons with full reports available, liver, spleen, and kidney samples were processed for paraffin embedding. In addition to routine H&E staining, in a selection of cases, 3-µm thin sections were stained according to Feulgen: hydrolysis in 1 N HCl for 30 min at 60 °C, rinsing in 1 N HCl at room temperature, staining with fresh Shiff reagent (Sigma, St. Louis, Mo.) for 45 min, and washing in tap water for 15 min. Slides were then counterstained with 1% Light Green (Sigma) for 2 min. Finally, the specimens were dehydrated in alcohol and mounted in Entellan (Merck, Darmstadt, Germany).

Immunohistochemistry

The following panel of antibodies was used: CD68 (clone PG-M1; DAKO, Glostrup, Denmark; dilution 1:100, microwave pretreatment) [11, 12], CK18 (clone M3; IDL, Sweden; dilution 1:10, pepsin digestion pretreatment) [13], and M30 (Roche Molecular Biochemicals, Germany; dilution 1:150, microwave pretreatment). M30, recognizing the epitope of interest, is an IgG_{2b} mouse monoclonal antibody, raised by immunization of Balb/c mice with CK18 fragments from cell culture supernatant from the WiDr CCL218 colon cancer cell line. The neo-epitope recognized by this antibody is formed by caspase cleavage at the sequence DALD-S, situated at a liberated C terminus of a CK18 fragment [10].

Sections of 3 µm were cut from the paraffin-embedded blocks, mounted on APES- (3-aminopropyltriethoxysilane; Sigma) coated slides, and air-dried overnight at 37 °C. For immunostaining, the sections were deparaffinized in xylene and rehydrated in a descending ethanol series. Endogenous peroxidase activity was blocked by immersion for 10 min in 3% hydrogen peroxide in methanol, after which the slides were rinsed in phosphate-buffered saline (PBS; pH 7.2–7.4). Samples for tests with the anti-cytokeratin antibody M3 needed pretreatment with 0.1% pepsin/0.1 N HCl (Sigma), whereas samples for treatment with the other antibodies were not enzymatically pretreated, but placed in a 0.1 M citrate solution (pH 6.0) at 90 °C in a microwave oven for 10 min and then cooled for 15 min in citrate solution at room temperature (RT). After preincubation with 1% bovine serum albumin (Sigma)/PBS for 10 min, the primary antibody was applied at the appropriate dilution for 1 h at RT. After washing in PBS, the secondary antibody (biotin-labeled goat anti-mouse Ig (1:400 diluted; DAKO) was applied for 45 min at room temperature. After washing in PBS, the slides were incubated with streptavidin conjugated with horseradish peroxidase (1:600; DAKO). After washing in PBS, peroxidase activity was detected with aminoethylcarbazole (AEC) staining solution (Zymed, South San Francisco, Calif.). Finally, sections were counterstained with Harris' hematoxylin and embedded in Kaiser's glycerin gelatin (Merck).

Terminal dUTP nick end labeling of fragmented DNA

Tissue sections were deparaffinized and rehydrated as described above. After blocking the endogenous peroxidase activity in 3% hydrogen peroxide/methanol, the sections were treated with proteinase K (20 µg/ml; Sigma) at 37 °C for 15 min, washed in PBS buffer, and incubated with TdT (0.3 units/ml; Boehringer Mannheim, Germany) and FITC-labeled 11-deoxyuridine triphosphate (dUTP-FITC; 20 mM; Boehringer Mannheim) in TdT buffer (30 mM TRIZMA base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). After 1 h at 37 °C, the slides were rinsed in 4×SSC (0.6 M NaCl, 0.06 M sodium citrate) for 5 min at RT, rinsed in PBS, incubated with the alkaline phosphatase converter antibody (alkaline phosphatase-labeled antibodies directed against FITC; Boerhinger Mannheim) for 30 min at 37 °C, and rinsed in PBS. The slides were developed for 15 min at 37 °C, using a specified substrate [14, 15], containing naphthol-AS-MX-phosphate/Fast Red TR (Sigma; red color). After rinsing in distilled water, the slides were counterstained in Harris' hematoxylin and embedded in Kaiser's glycerin gelatin.

Scoring of M30 immunostaining

A two-person consensus score of the immunostaining was established and restricted to the localization of the epitope. Scoring of the M30-positive structures was performed by a semiquantitative estimation of the cells and fragments positive for this antibody. Analyses of the proportion of stained structures within each section yielded three groups: no fragments or reactive cells (score 0), detectable fragments or immunopositive cells (score 1), and fragments in all microscopic fields (score 2). All microscopic fields were examined by ×40 enlargement. When fragments contained hematoxylin-reactive material (DNA) this was noted. Differences in staining intensity were not evaluated.

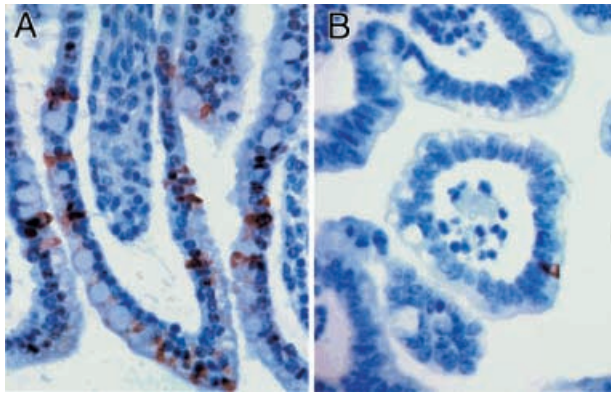

Figure 1. Immunostaining for M30 in the small intestines of a fetus of 28 (*A*) and 18 weeks (*B*). As shown, the numbers of apoptotic cells vary at different fetal ages. The immunostained cells show strong cytoplasmic staining; some in *A* show signs of cytoplasm detachment from the environment: the nucleus of those cells becomes expelled toward the lumen of the gut.

Results

The fetus

A low number of epithelial cells were positive for M30. However, in a few cases, an increase in M30-positive epithelial cells could be found in the mucosa of the gastrointestinal tract (fig. 1), and in the bronchial epithelium of the lung. Whereas epithelial linings of many organs were not often M30 positive, the supportive stroma of in-

Table 1. M30 immunostaining of fetal liver and spleen (n = 21).

		IHC score		
		0	1	2
Liver	hepatocytes	20 (93%)	1 (7%)	0
	fragments in sinusoids	18 (85%)	3 (15%)	0
	macrophages	0	8 (39%)	13 (61%)
Spleen	fragments in red pulp	0	9 (43%)	12 (57%)
	macrophages	0	0	21 (100%)

Score 0, no fragments or reactive cells; score 1, detectable fragments or immunopositive cells; score 2, fragments in all microscopic fields.

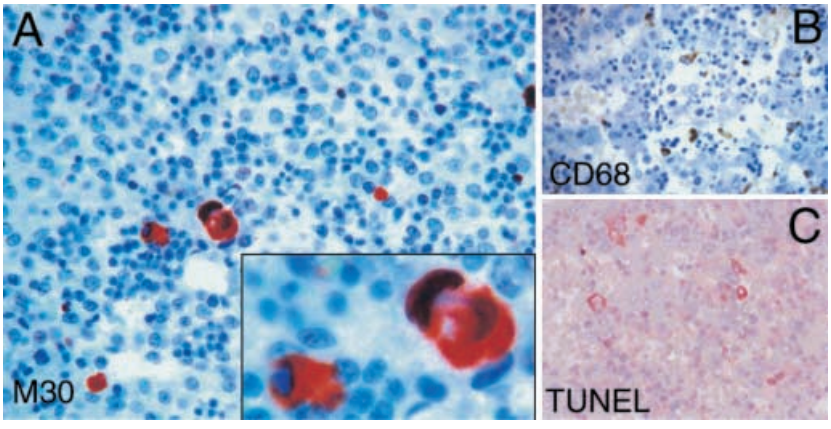

Figure 2. Immunohistochemistry with M30 (*A*) and CD68 (*B*), and TUNEL assay (*C*) in a liver of a 12-week fetus. (*A*) The M30 immunostaining shows numerous immunopositive macrophages in the hepatic sinusoids. The number of free M30-positive fragments is low. (*B*) Staining for CD68 shows that cells in the hepatic sinusoids are macrophages. (*C*) The TUNEL assay shows that those cells with faint cytoplasmic staining may be from ingested apoptotic nucleosomal particles. A few apoptotic hepatocytes with TUNEL-positive nuclei are also present.

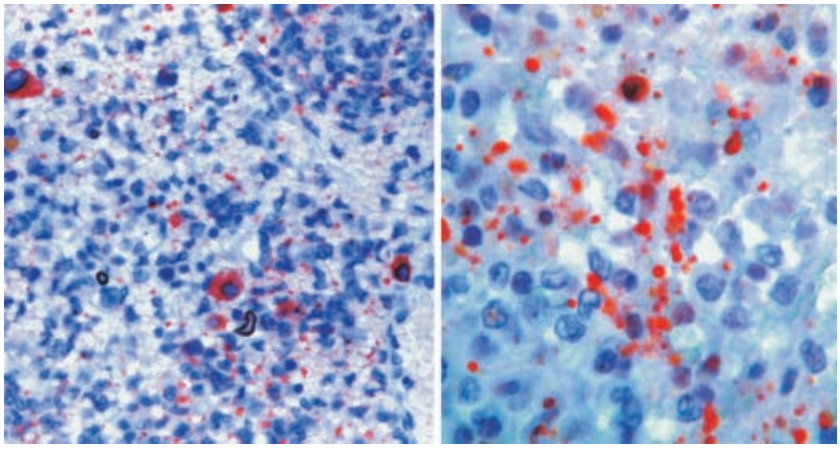

Figure 3. Expression of the M30 epitope in the spleen of a fetus of 12 (*A*) and 28 weeks (*B*). In the splenic sinusoid of the red pulp there are many M30-positive fragments and macrophages are also present loaded with M30-positive particles. As shown at higher magnification (*B*), the spleen in the older fetus becomes repleted with epithelial apoptotic fragments.

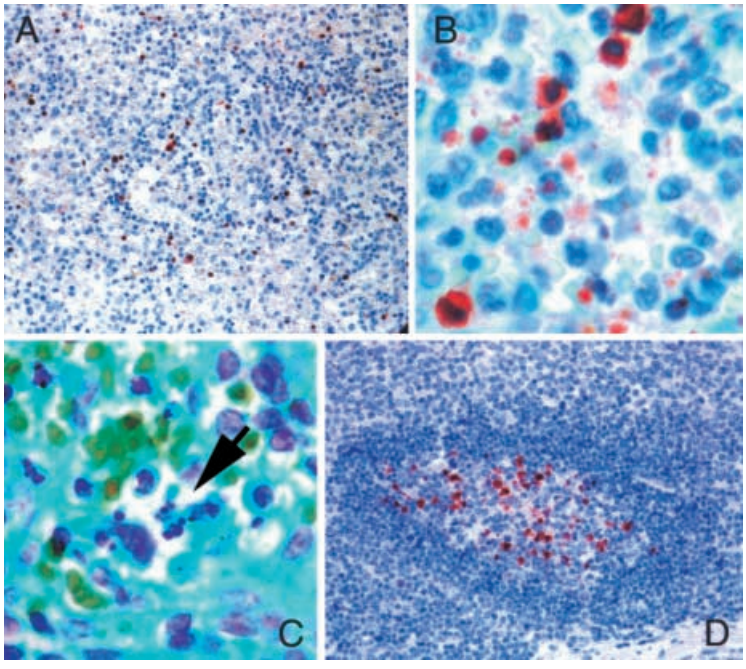

Figure 4. M30 immunostaining in the spleen of an adult patient with metastasized colorectal carcinoma, showing splenic sinusoids (*A*, *B*), staining after the Feulgen reaction (*C*), and M30 staining in the germinal center of the secondary lymph follicle (*D*). (*A*) Many DNA-containing M30-positive fragments are present in the splenic sinusoids next to numerous fragments. (*B*) At higher magnification, the complete and the nuclear fragments vary in size. (*C*) After Feulgen staining, the hematoxylin-reactive material found in those fragments appears to contain DNA as indicated by a purple staining. Disintegrating apoptotic nuclei can also be found (arrow). (*D*) From the same patient, these DNA-containing particles are also found to be situated in the germinal center of secondary lymph follicles of the periarteliolar lymphoid sheath of the spleen.

testines, esophagus, kidneys, heart, lung, and stomach showed the presence of M30-positive fragments, either as free particles or in the cytoplasm of macrophages. These fragments varied in size from the limits of visibility (when examined by light microscopy) to almost 10 µm, the size of an erythrocyte, and were irregularly shaped. In almost all cases, the M30-positive fragments outside macrophages could be detected in the lumen of blood or lymph vessels, at constant short distances from the mucosal surface. The fragments did not react with antibodies against CK18 and were not visible in routine H&E-stained sections.

CD68-positive macrophages, also immunoreactive with M30, were often present outside vascular structures in the

Table 2. M30 immunostaining of adult spleen.

Class	Category	n	No fragments	Fragments	Fragments+DNA
1	respiratory failure/cardiac decompensation	28	6 (21%)	20 (71%)	2 (7%)
2	chronic inflammatory processes	12	0 (0%)	10 (83%)	2 (17%)
3	localized primary cancer	15	1 (7%)	11 (73%)	3 (20%)
4	disseminated cancer	16	3 (19%)	0 (0%)	13 (81%)
5	not specified	39	20 (51%)	17 (44%)	2 (5%)
	Total	110	30 (27%)	58 (53%)	22 (20%)

stroma of several organs, including intestines, lung, kidneys, spleen, and liver (fig. 2). Very similar M30-positive macrophages were also found in the dermis, especially in the peripheral regions of the upper and lower extremities. In the spleen, accumulation of M30-positive small cytoplasmic fragments was seen in the vascular spaces of the red pulp. Only incidental macrophages with M30-immunoreactive material in their cytoplasm were seen, whereas in the sinusoids of the liver, the opposite situation occured (table 1, Figs. 2, 3). Despite the fact that the tissue had sometimes undergone autolytic changes, the majority of apoptotic fragments remained immunoreactive for M30.

There was a wide distribution of TUNEL-positive cells in the fetus at different stages of development. The labeling was not only confined to the nucleus. In the spleen, much but weak cytoplasmic nick-end reactivity was detected. Most positive cells were found in the stroma of several organs and in the periphery of the limbs. Double-immunostaining experiments showed that the areas with TUNEL-positive cells coincided with those containing M30-positive cells. In epithelia, the number of TUNEL-positive cells was also low. In the intestines, cells at the tip of the villi were stained, although these cells did not have the morphological criteria of apoptosis. When examined by double immunostaining, there were macrophages which were positive with both M30 and the TUNEL assay. In the liver of older fetuses, TUNEL-positive Kupffer cells could be detected in the sinusoids (fig. 2C). In the TUNEL experiments, separating TUNEL-positive cells and fragments from background staining in the spleen was difficult. Autolytic and necrotic tissues showed a strong homogeneous background staining.

Adults

The results of 110 autopsy cases could be broadly classified into five categories (table 2). In adult tissues, non-phagocytosed fragments were rarely detected in the interstitium of the tissues, and only a scattering of M30-positive fragments were normally detected in the red pulp of the spleen. Only in classes 2, 3, and 4 (table 2) was an increase in M30-positive apoptotic epithelial fragments found. In these patients, M30-positive fragments could also be detected in the blood and lymph vessels in the diseased organs. Patients with chronic inflammatory disease (class 2) showed extensive accumulation of apoptotic fragments in the red pulp of the spleen, whereas patients with respiratory failure or cardiac decompensation showed less or no fragments. The fragments showed no immunoreactivity with antibodies directed against CK18. When the number of M30-positive fragments was very high in the spleen, a scattering of fragments could be found in the liver. In one case, M30-positive fragments were observed in bile thrombi present in bile canaliculi of the liver. This was from a patient with metastasized adenocarcinoma to the liver with many apoptotic tumor cells. In the kidneys, fragments could not be observed in any of the pathological situations examined. A remarkable finding was that 13 of 16 patients with metastasized cancer (class 4) showed a pronounced accumulation of M30-positive fragments, with hematoxylin-reactive small pycnotic nuclear fragments in the spleen (fig. 4A, B, table 3), but not detected outside the spleen. The hematoxylin-reactive nuclear fragments were also positive in the Feulgen reaction, indicating the presence of DNA (fig. 4C). The overall size of these fragments in this patient group (class 4) was also large and, in a few cases, the DNA-containing M30-positive fragments were found in the germinal center of periarteriolar lymphoid sheaths (PALS; see also fig. 4D). The DNA-containing fragments were only seen in 9/94 cases other than metastasized cancer.

Discussion

We examined the phagocytosis and clearance of apoptotic epithelial cells during human development and in adulthood. The cytoskeleton is affected early in the apoptotic cascade [16–20]. Cytokeratin filament aggregates can be detected in apoptotic bodies and, when phagocytosed, in macrophages [17, 19, 20], but apoptotic cells are difficult to identify because they are surrounded by normal, 'vital' epithelial cells which are also positive for the cytokeratins. However, the apoptotic cells can be identified by immunostaining with M30 antibody, because the surrounding normal cells are then completely negative. Immunohistochemical staining with the M30 antibody has now revealed that the result of apoptosis in epithelial cells

Table 3. M30 immunostaining of DNA-containing fragments.

	Class 4	Other classes	Total
Fragments + DNA	13	9	22
(No) fragments - DNA	3	85	88
Total	16	94	110

Sensitivity = (13/16) × 100 = 81%
Specificity = (85/94) × 100 = 90%

can be detected as scattered individual fragments. During adulthood, in normal situations, apoptotic fragments were not found by light microscopy, but in situations with increased apoptotic activity, M30-positive fragments and macrophages could be found. From the findings presented in this study, we can evaluate several aspects of the apoptotic cytokeratin fragments.

Lack of splenic apoptotic fragments with anti-cytokeratin antibodies

After induction of apoptosis in epithelial cell cultures, a positive reaction in the cytoskeleton can be observed with most cytokeratin antibodies [19, 20]. We observed both CK18 and M30 reactivity under similar conditions [10]. However, in M30-positive cell fragments in vessel spaces and in the spleen, no cytokeratin immunoreactivity was observed with the commercially available antibodies. The finding that the M30-positive fragments do not react with ordinary cytokeratin antibodies can be explained by the different locations of the epitope on the CK18 filament protein: the M30 neo-epitope is created during early apoptotic cleavage and is situated near the C terminus of the CK18 protein [10]. We demonstrated earlier that in late apoptotic cells, the cleavage process results in a CK18 fragment of approximately 20 kDa, in which the M30 epitope is retained but the M3 epitope and other cytokeratin epitopes are not [10].

The splenic M30-positive material may indicate accumulation of apoptotic cellular debris in the spleen

Enlargment of the spleen in sepsis is generally believed to be caused by the accumulation of cells and cellular debris. Normal breakdown of erythrocytes takes place in the spleen, followed by transportation to the liver for further processing and final excretion into the bile. In a balanced situation, the apoptotic bodies are removed by macrophages. Sometimes, we observe a weakly positive cytoplasmic staining for M30 in macrophages. In this situation, the spleen only shows a few scattered M30-positive fragments (class 1 of the autopsy cases). However, when the apoptotic activity is higher and the load of fragments is high in chronic inflammation such as ulcerative colitis or in patients with metastasized malignant epithelial tumors, the affected tissues show an increased expression of M30-positive apoptotic cells. In those cases, a huge accumulation of M30-positive fragments was also found in the red pulp of the spleen. Clearance by the spleen is obviously effective, because in the majority of the adult autopsy analyses, very few fragments could be detected in the liver and none in the kidney. However, an overload of the phagocytic clearance capacity has previously been observed when massive apoptosis in the liver was induced by administration of anti-Fas antibodies to mice [21].

Whereas an accumulation of macrophages was seen in the adult spleen under pathological conditions, the number of M30-positive macrophages in the spleen is low during fetal development. However, in fetal liver, a large number of M30-positive macrophages were detected. In addition, very few free M30-positive fragments were detected in the fetal hepatic sinusoids. This observation might be understood if one considers the macrophages as transport vehicles from the spleen to the liver in fetal growth, development, and recycling of cell constituents.

Epithelial apoptotic fragments in the spleen may play a role in the development of immunological tolerance for or rejection of neo-epitopes

The accumulation of M30-positive fragments in the spleen during development in the fetus may possibly act as a normal procedure to induce tolerance for this and other neo-epitopes. Impaired completion of tolerance induction, or the formation of other epitopes during apoptosis in the adult may initiate autoimmune reactions. The observation of M30-positive DNA-containing apoptotic bodies in the germinal center of the PALS system of the spleen in five of our patients might be interpreted in this context. In support of this conclusion, observations on apoptotic keratinocytes are of interest. Two populations of surface structures have been shown to exist on such keratinocytes: small buds containing fragmented endoplasmic reticulum and ribosomes, and large buds (apoptotic bodies) containing nucleosomal DNA and nuclear ribonucleoproteins [22]. In those studies, the membrane-buds, surrounding the keratinocytes undergoing apoptosis, were presumed to contain concentrates of neo-epitopes or autoantigens and to have an important function in antigen presentation to the immune system. In systemic lupus erythematosus, there is evidence that defects in the apoptotic process are linked to the pathogenesis of the disease, and in SLE patients, proteins cleaved by the ICE family of proteases during apoptosis could result in the formation of autoantibody products [23–25]. Furthermore, a subpopulation of macrophages involved in the phagocytic clearance of apoptotic cells in rat colon tumors expresses cell surface molecules associated with antigen presentation and stimulation of naive splenocytes [26].

Role of DNA-containing apoptotic fragments in late metastases

In the category of patients with metastatic tumors, a preferential occurrence of DNA-containing M30-positive fragments in the spleen was found. In all other categories, the occurrence of the DNA-containing fragments was much lower and, in most cases, absent. Because those fragments were also larger, the most reasonable interpretation is that these fragments constitute apoptotic bodies, containing nuclear fragments, which have escaped local phagocytic clearance. Recent experiments [27] have shown that DNA remnants within apoptotic bodies can be transferred to another cell where they are integrated into the recipient DNA, as demonstrated by cocultivation of apoptotic bodies of Epstein-Barr virus (EBV)-carrying cells with EBV-negative human fibroblasts, macrophages, and bovine aortic endothelial cells. This resulted in expression of EBV-encoded genes in the recipient cells at high frequency and the transferred DNA was stable over time. Recently, oncogenes present in apoptotic bodies were also shown to be horizontally transferred to eukaryotic cells resulting in aneuploidy and accumulation of genetic changes necessary for tumor formation [28]. The accumulation of DNA-containing M30-positive fragments in the spleen of patients with metastatic malignancies, found in the present study, may similarly lead to transfer and integration of tumor DNA remnants from those apoptotic bodies into other normal cells.

In conclusion, our study visualized the existence of remnants of the apoptotic process by a simple immunochemical assay. When the apoptotic load is too high, phagocytic clearance of apoptotic bodies can apparently become inefficient and fragments may remain intact before they are transported to the spleen where they are filtered out of the blood. Detection of M30-positive fragments in the bloodstream may reflect the activity of disease or therapy.

1 Saunders J. and Fallon J. (1966) Cell death in morphogensis. In: Major Problems in Developmental Biology, pp. 289–314, Locke M. (ed.), Academic Press, New York
2 Prindull G. (1995) Apoptosis in the embryo and tumorigenesis. Eur. J. Cancer **1:** 116–123
3 Hinrichsen K. (1985) The early development of morphology and patterns of the face in the human embryo. In: Advances in Anatomy of Embryologic Biology, pp. 98–101, Beck F. H. W., Kriz W., Ortmann R., Pauly J. E. and Schieber T. M. (eds), Springer, Berlin
4 Goldmann A., Baker M., Peddington R. and Herold R. (1983) Inhibition of programmed cell death in mouse embryonic palate in vitro by cortisone and phenytoin: receptor involvement and requirement of protein synthesis. Proc. Soc. Exp. Biol. Med. **174:** 239–243
5 Pexieder T. (1975) Cell death in the morphogenesis and teratogenesis of the heart. Adv. Anat. Embryol Cell. Biol. **51:** 5–99
6 Saunders J. (1966) Death in the embryonic system. Science **154:** 604–612
7 Kerr J. (1971) Shrinkage necrosis: a distinct mode of cellular death. J. Pathol. **105:** 13–20
8 Kerr J. and Harmon B. (1994) Definition and incidence of apoptosis: an historical perspective. In: Apoptosis: The Molecular Basis of Cell Death, pp 79–108, Tomei L. and Cope F. (eds), Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N. Y.
9 Bosman F., Visser B. and Oeveren J. van (1996) Apoptosis: pathophysiology of programmed cell death. Pathol. Res. Pract. **192:** 676–683
10 Leers M. P. G., Kolgen W., Bjorklund V., Bergman T., Tribbick G., Persson B. et al. (1999) Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. J. Pathol. **187:** 567–572
11 Holness C. and Simmons D. (1993) Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood **81:** 1607–1613
12 Falini B., Flenghi L., Pileri S., Gambacorta M., Bigerna B., Durkop H. et al. (1993) PG-M1: a new monoclonal antibody directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. Am. J. Pathol. **142:** 1359–1372
13 Bonfrer J. M., Groeneveld E. M., Korse C. M., Dalen A. van, Oomen L. C. and Ivanyi D. (1994) Monoclonal antibody M3 used in tissue polypeptide-specific antigen assay for the quantification of tissue polypeptide antigen recognizes keratin 18. Tumour Biol. **15:** 210–222
14 Speel E. J., Jansen M. P., Ramaekers F. C. S. and Hopman A. H. (1994) A novel triple-color detection procedure for brightfield microscopy, combining in situ hybridization with immunocytochemistry. J. Histochem. Cytochem. **42:** 1299–1307
15 Speel E. J., Herbergs J., Ramaekers F. C. S. and Hopman A. H. (1994) Combined immunocytochemistry and fluorescence in situ hybridization for simultaneous tricolor detection of cell cycle, genomic, and phenotypic parameters of tumor cells. J. Histochem. Cytochem. **42:** 961–966
16 Engeland M. van, Nieland L. J., Ramaekers F. C. S., Schutte B. and Reutelingsperger C. P. (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry **31:** 1–9
17 Caulin C., Salvesen G. S. and Oshima R. G. (1997) Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. J. Cell Biol. **138:** 1379–1394
18 Ku N. O., Liao J. and Omary M. B. (1997) Apoptosis generates stable fragments of human type I keratins. J. Biol. Chem. **2:** 33197–33203
19 Tinnemans M. M., Lenders M. H., Velde G. P. ten, Ramaekers F. C. S. and Schutte B. (1995) Alterations in cytoskeletal and nuclear matrix-associated proteins during apoptosis. Eur. J. Cell Biol. **68:** 35–46
20 Engeland M. van, Kuijpers H. J., Ramaekers F. C. S., Reutelingsperger C. P. and Schutte B. (1997) Plasma membrane alterations and cytoskeletal changes in apoptosis. Exp. Cell Res. **235:** 421–430
21 Ogasawara J., Watanabe-Fukunaga R., Adachi M., Matsuzawa A., Kasugai T., Kitamura Y. et al. (1993) Lethal effect of the anti-Fas antibody in mice. Nature **364:** 806–809
22 Casciola-Rosen L., Anhalt G. and Rosen A. (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J. Exp. Med. **179:** 1317–1330
23 Casciola-Rosen L., Anhalt G. and Rosen A. (1995) DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. J. Exp. Med. **182:** 1625–1634
24 Nicholson D. W., Ali A., Thornberry N. A., Vaillancourt J. P., Ding C. K., Gallant M. et al. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature **376:** 37–43

25 Tewari M., Quan L.T., O'Rourke K., Desnoyers S., Zeng Z., Beidler D. R. et al. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell **81:** 801–809
26 Henry F., Bretaudeau L., Barbieux I., Meflah K. and Gregoire M. (1998) Induction of antigen presentation by macrophages after phagocytosis of tumour apoptotic cells. Res. Immunol. **149:** 673–679
27 Holmgren L., Szeles A., Rajnavolgyi E., Folkman J., Klein G., Ernberg I. et al. (1999) Horizontal transfer of DNA by the uptake of apoptotic bodies. Blood **93:** 3956–3963
28 Bergsmedh A., Szeles A., Henriksson M., Bratt A., Folkman M. J., Spetz A. L. et al. (2001) Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc. Natl. Acad. Sci. USA **98:** 6407–6411