Cell Tissue Res (1994) 278:549–556

Cell & Tissue Research

Distinction of apoptotic and necrotic cell death by in situ labelling of fragmented DNA

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Received: 13 September 1993 / Accepted: 13 May 1994

Abstract. The occurrence and spatial distribution of intracellular DNA fragmentation was investigated by in situ 3′end labelling of DNA breaks in K562 cells treated in such a way to cause either apoptotic or necrotic cell death. The localisation of DNA breaks was examined by confocal laser microscopy and compared with the electron-microscopic appearance of the cells. In addition, the number of cells with fragmented DNA was counted and compared with the number of dead cells, as determined by the nigrosin dye exclusion test. Apoptosis was induced by cultivation of the cells in the presence of actinomycin D. Cells undergoing apoptosis were characterised by massive intracellular DNA fragmentation that was highly ordered into successive steps. Cells in early stages of the apoptotic process had DNA breaks diffusely distributed in the entire nucleus, except the nucleolus, with crescent-like accumulations beyond the nuclear membrane. In the more advanced stages, the nucleus was transformed into many round bodies with intense labelling. Intracellular accumulations of fragmented DNA corresponded exactly to electron-dense chromatin seen in the electron microscope, whereas diffuse DNA breaks had no morphological correlate at the ultrastructural level. In necrosis induced by ionomycin, NaN_3, or rapid freezing combined with thawing, no DNA fragmentation occurred at the onset of cell death, but appeared 24 h later. This fragmentation was not characterised by a unique morphology, but represented the breakdown of the chromatin in the configuration remaining after cell death. Therefore, apoptosis is characterised by DNA fragmentation that proceeds in a regular orderly sequence at the beginning of cell death, and can be detected by in situ 3′end labelling of DNA breaks.

Key words: Apoptosis – Necrosis – Cell death – DNA fragmentation – Endogenous nuclease – Actinomycin D – Ionomycin – Confocal laser microscopy

Introduction

Cells whose energy-providing systems or whose plasma membrane integrity has been irreversibly damaged die inevitably by ion and fluid imbalance in an inorderly fashion (Bowen and Bowen 1990). The loss of functionally intact tissue followed by the inflammatory reaction and scarring is deleterious, and organisms try to avoid or minimise this type of cell loss. In a large number of instances where cell death occurs, another type of cell death has been described called apoptosis, which is characterised by its uniform morphological appearance, regardless of the kind of tissue involved or the eliciting stimulus (Arends and Wyllie 1991).

Apoptotic cells are morphologically characterised by shrinkage of the cytoplasm and condensation of the nuclear chromatin. Subsequently, the nucleus and cytoplasm break into many so-called apoptotic bodies, with or without nuclear fragments, which are rapidly phagocytosed by the neighbouring cells or by macrophages (Kerr et al. 1972; Wyllie et al. 1984). No signs of damage to the cell organelles have been detected by ultrastructural or histochemical methods (Kerr 1965; Pipan and Sterle 1979). Apoptosis can be elicited by a physiological stimulus that is not per se harmful and that causes death to only a specific population of cells. The dying cells are not contiguous but scattered in the tissue; entering the apoptotic pathway is asynchronous and continues for days after an acute eliciting event (Butyan 1991). These characteristics have led to the hypothesis that the organism eliminates cells that are no longer needed or that are potentially harmful to the organism by apoptosis, i.e. apoptosis is apparently the counterpart of mitosis, regulating the number of differentiated cells (Kerr et al. 1972). The uniform appearance of cells undergoing apoptosis,

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regardless of the causative stimulus and the non-toxic nature of some stimuli, indicates a gene-directed process of cellular self-destruction with active participation by the dying cell. The intracellular pathways of apoptosis are, however, not completely understood.

The best defined biochemical event in apoptotic cells is the cleavage of the nuclear genome at the linker regions between the nucleosomes; this yields DNA fragments with multiples of about 200 base pairs (Arends et al. 1990). Fragmented DNA has also been isolated from necrotic cells. However, this seems not to be an early event in the cell death process, as is the case in apoptosis, but represents a secondary process of unspecific degradation of the chromatin, probably by lysosomal proteins (Afanas'ev et al. 1986; Collins et al. 1992).

Because of internucleosomal fragmentation, the DNA isolated from apoptotic cells shows a characteristic "DNA ladder" on agarose gel electrophoresis, demonstrating a regular fragmentation of the nuclear genome. However, this procedure reflects the sum of the DNA status of all cells in the tissue extract and, thus, the question arises whether the specific identification of cells undergoing apoptosis can be demonstrated. This would allow an exact quantification not only of the number of apoptotic cells, but also of the time course and spatial distribution of the DNA fragmentation. A precise identification of apoptosis at the single cell level would furthermore allow simultaneous immunhistochemical studies of other intracellular proteins involved in this process or the definition of a specific subpopulation of cells. In addition, the type of DNA fragmentation in apoptotic cells and the type in necrotic cells could be compared.

We used an in situ 3'end labelling method for the detection of DNA breaks in single cells that were induced to undergo either apoptotic or necrotic cell death. Morphological characteristics and the time points of the appearance of DNA fragmentation were investigated. Parts of this study have been published in abstract form (Kressel and Groscurth 1993).

Materials and methods

Cell culture

K562 cells were cultivated in suspension in RPMI-1640 medium (Life Technologies, Basel, Switzerland) supplemented with 200 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum. Cells were maintained in plastic tissue-culture flasks (25 cm^2; Nunc, Roskilde, Denmark) at 37°C in a 5% CO_2/95% air atmosphere and harvested under logarithmic growth at a density of 1×10^6 per ml for experiments.

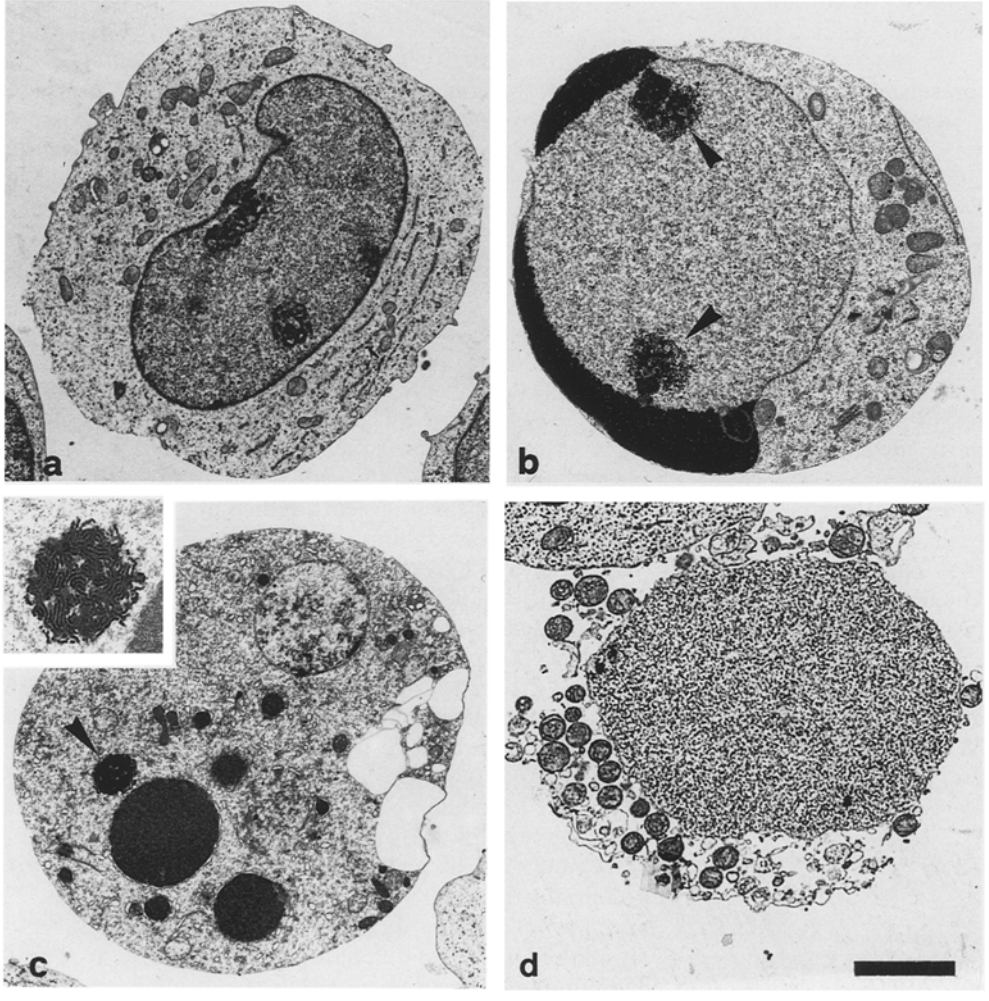

Fig. 1a–d. Transmission electron-microscopic appearance of K562 cells. **a** Cells from control cultures. **b, c** Apoptotic cells cultured for 24 h with actinomycin D; early and late phases, respectively. *Arrowheads* Altered nucleoli. *Inset* in **c** shows higher magnification of the remaining nucleolus. **d** Necrotic cell cultured for 3 h with ionomycin. *Bar*: 3 µm **(a, c)**, 2 µm **(b)**, 3 µm **(d)**

Experimental protocol

Stock solutions of 1 mg/ml ionomycin (Sigma, Buchs, Switzerland) in dimethylsolfoxide and 1 mg/ml actinomycin D (Sigma) in methanol were prepared. Apoptotic cell death was induced by adding actinomycin D to the incubation medium at a final concentration of 1 μg/ml. Necrosis was produced in three different ways. (1) Cells were cultivated in the presence of ionomycin at a final concentration of 3 μg/ml. (2) Cells were grown in the presence of NaN_3 at a final concentration of 460 mM. (3) Cells were subjected to three cycles of rapid freezing in liquid nitrogen and thawing (37°C) and then further cultivated. Aliquots were taken after 3 and 24 h. Cell viability was determined by the nigrosin dye exclusion test (Wotring et al. 1985) and the cells were fixed in suspension for either electron microscopy or in situ DNA 3′end labelling.

Transmission electron microscopy

Cells were prefixed in 2.5% glutaraldehyde plus 0.8% paraformaldehyde (0.05 M sodium cacodylate buffer) for 30 min, post-fixed in 1% OsO_4 plus 1.5% K_4 $Fe(CN)_6$ and embedded in Epon. Ultrathin sections (approximately 50 nm) were contrasted with uranyl acetate and lead citrate, and examined with a TEM 420 (Philips).

In situ labelling of DNA breaks

Cells were fixed in 3% paraformaldehyde dissolved in 80 mM KPIPES (pH 7.5) (Sigma) supplemented with 5 mM EGTA and 2 mM $MgCl_2$, for 1 h. Subsequently, cytospin preparations were made on slides pre-coated with 3-aminopropyltriethoxysilane (Sigma) (Maddox and Jenkins 1987). Cells were quenched for 15 min in freshly prepared $NaBH_4$ (1 mg/ml) (Fluka, Buchs, Switzerland) in phosphate-buffered saline (PBS), pH 8, and after washing with PBS, they were permeabilised for 4 min in 0.1% Triton X-100 in PBS. After 20 min incubation in PBS plus 1% normal goat serum (NGS), the slides were washed in twice distilled water and incubated in the reaction mixture for 4 h at 37°C.

The reaction mixture consisted of 0.5 units/μl terminal transferase, 2.5 mM $CoCl_2$ and 10 μM biotin-16-dUTP in 250 mM potassium cacodylate, 25 mM TRIS buffer (pH 7.2) (all reagents from Boehringer Mannheim, Rotkreuz, Switzerland). The slides were then washed in distilled water and incubated for 30 min in streptavidin-fluorescein-isothiocyanate (Bio Science Products, Emmenbrücke, Switzerland) diluted 1:50 in PBS containing 1% NGS. Finally, the specimens were mounted in Vectashield (Vector Laboratories, Burlingame, USA) and stored at 4°C for up to 4 weeks.

For negative controls, either the enzyme terminal transferase or $CoCl_2$ was omitted from the reaction mixture. For positive controls, slides were pre-treated overnight with 0.4 units/μl DNAseI in 50 mM TRIS (pH 7.9), supplemented with 5 mM $MgCl_2$, 10 mM mercaptoethanol, 50 μg/ml bovine serum albumin (Gazit and Cedar 1982) and then labelled.

Confocal laser microscopy

Slides were examined with a LSM-II (Zeiss). Fluorescence and differential interference contrast (DIC) images were recorded from each specimen. For the fluorescence micrographs, serial optical sections were taken typically at 0.9-μm depth increments and digitally

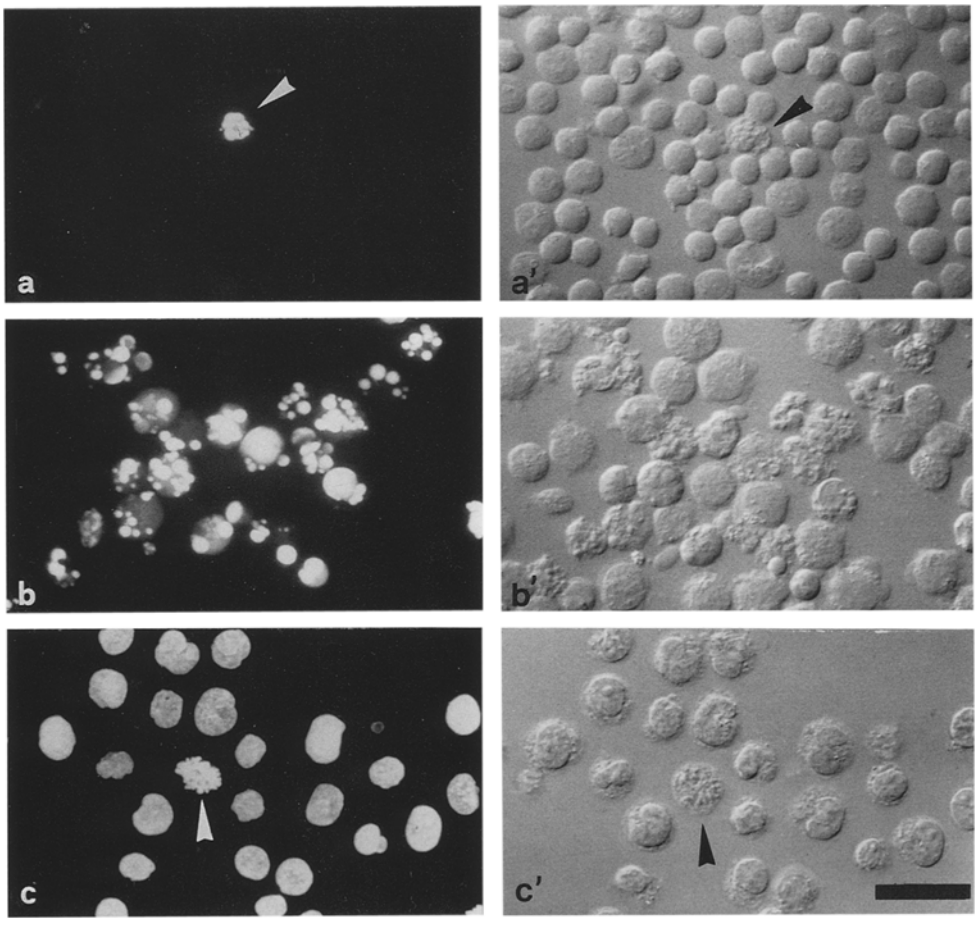

Fig. 2a–c, a′–c′. In situ 3′end labelling after induction of apoptosis. Fluorescence image by confocal microscopy **(a–c)** and the corresponding interference contrast image **(a′–c′)** in cells cultured for 24 h. **a, a′** Only one labelled cell (*arrowhead*) can be seen in controls without actinomycin D. **b, b′** Increased numbers of labelled cells following treatment with actinomycin D. **c, c′** All cells are labelled after preincubation of untreated K562 cells with DNAseI. *Arrowhead* A mitotic figure. *Bar*: 19 μm **(a)**, 14 μm **(b)**, 11 μm **(c)**

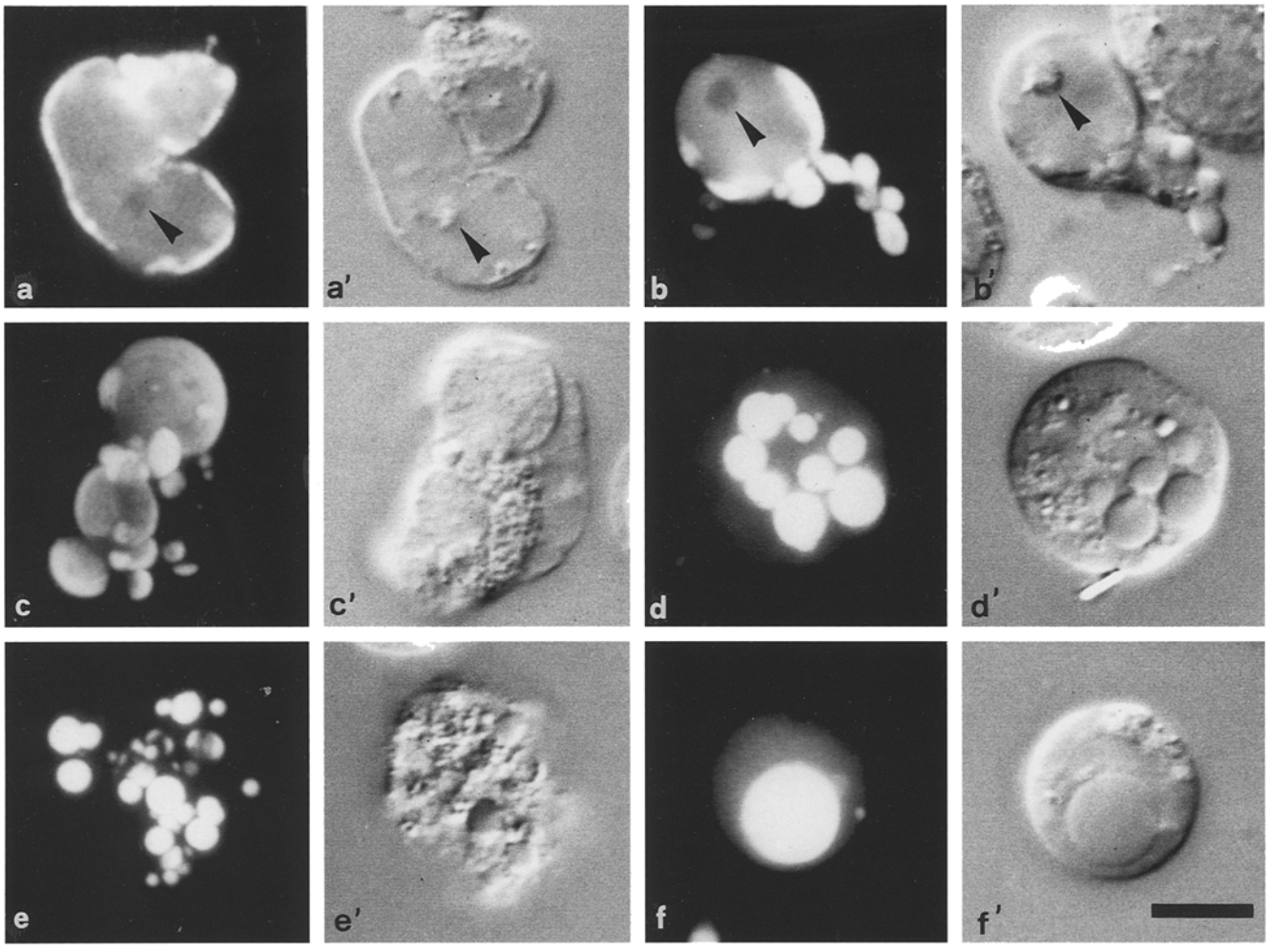

Fig. 3a–f, a′–f′. Morphological appearance of DNA fragmentation in apoptotic cells. Fluorescence images **(a–f)** and corresponding interference contrast images **(a′–f′)**. **a, a′** Nuclear boundaries outlined by an intensely labelled rim, whereas the interior shows a diffuse homogeneous signal. Neither the nucleolar region (*arrowhead*) nor the cytoplasm are labelled. **b, b′** Similar to **a**, but the nucleus is rounded up with peripheral crescents directly beyond the nuclear margins, from which intensely fluorescent bodies pinch off in the direction of the cytoplasm. The nucleolar region does not stain (*arrowhead*). **c, c′** Early fragmentation of the nucleus into separate bodies; diffuse labelling of the interior and peripheral crescents. The cytoplasm remains unlabelled. **d, d′** Fragmentation of the nucleus into many small intensely fluorescent homogeneous bodies. Note the diffuse labelling of the cytoplasm between the nuclear fragments. **e, e′** The nucleus is fragmented into many small bodies; there is no labelling of the cytoplasm. Some of the smaller fragments are half-moon shaped. **f, f′** DNA fragmentation and nuclear condensation but with no concomitant fragmentation of the nucleus into many small bodies. The cytoplasm also shows diffuse fluorescence. *Bar*: 9 μm

superimposed. This permitted an "all in focus" image of all the fluorescence within a single cell and allowed a three-dimensional reconstruction of the spatial distribution of the label. Subsequently, DIC images and fluorescence micrographs were digitally superimposed, in order to correlate the fluorescence with its cellular localisation. Except for adjustments of contrast and brightness, no further image processing was performed. When comparing the fluorescence intensity, care was taken to use the same contrast and brightness settings for all experiments. The total number of cells in the DIC image and the percentage of fluorescent cells was calculated for each experiment by random selection of four different fields with the 40 × objective and zoom factor 30. Values were expressed as the mean percentage of labelled cells from three independent experiments and the standard error of the mean.

Results

Apoptotic cell death

At the ultrastructural level, K562 cells from control cultures had a large indented nucleus with abundant euchromatin, little marginally condensed heterochromatin and one or more prominent nucleoli (Fig. 1 a). After culture with actinomycin D for 24 h, cells with all the criteria for apoptotic cell death were frequently observed (Fig. 1 b, c). The alterations in apoptotic cells involved the nucleus, whereas the cell organelles seemed not to be altered, except for the occasional vacuolisation of the rough endoplasmatic reticulum (ER) (Fig. 1 c). In apoptotic cells with unfragmented nuclei, the nuclear chromatin was segregated into sharply circumscribed dense masses and a clear matrix, in which euchromatin and heterochromat-

in were no longer discernible. The dark chromatin always lay beyond the nuclear envelope and was not separated from it by intervening matrix. In advanced stages, only nuclear fragments were found in the cells (Fig. 1 c), apparently originating by budding off from the original nucleus (data not shown). In large unfragmented nuclei, the electron-dense masses made up only a small rim at the nuclear periphery, whereas in smaller nuclear fragments, they successively increased to a more half-moon shape. The nucleolus was transformed into an aggregate of granular and thread-like structures that, after fragmentation of the nucleus, lay freely in the cytoplasm (Fig. 1 c).

In situ DNA 3′end labelling of these cultures revealed a distinct proportion of cells with intense fluorescence, whereas the remaining cells showed no labelling, with almost no background staining (Fig. 2 a, b). No fluorescence was detected after omission of the enzyme terminal transferase from the reaction mixture, nor was any seen when the reaction mixture lacked $CoCl_2$. In contrast, if slides were incubated with DNAseI after the fixation and permeabilisation step but prior to the labelling procedure, all nuclei including mitotic figures were heavily labelled (Fig. 2 c) and the nuclei were outlined by an intensely labelled ring immediately along the nuclear margin. Thus, DNAseI and terminal transferase were able to penetrate the nuclei of all cells.

Although only a few labelled cells were found in untreated cultures (Fig. 2 a), the percentage increased dramatically after 24 h of culturing in the presence of actinomycin D (Fig. 2 b). The cells were examined by confocal laser microscopy to gain more information about the intracellular distribution of the fluorescence. Superimposing DIC with fluorescence images allowed an exact correlation of the label to nucleus or cytoplasm, as the localisation and margins of the nucleus or nuclear fragments were visible in the DIC image with good resolution. By this procedure, different morphological categories of apoptotic cells could be distinguished. In the first group, which represented about 10% of labelled cells, the nucleus was outlined by intense fluorescence directly beyond its margin. When the nucleus was large and irregular or kidney-shaped, this fluorescence was observed as an even ring around its periphery (Fig. 3 a). In most instances, however, the nucleus appeared rounded and the fluorescence had the shape not of a band, but of bright crescents that were either aligned with, or clearly separated from, each other (Fig. 3 b). The number and size of these crescents per nucleus varied; some had only two large crescents at the opposite poles, some had numerous crescents irregularly distributed around the whole circumference and contiguous with one another. Inside the nucleus, a homogeneous diffuse labelling could clearly be resolved; this was limited to the nuclear region. In addition, a round non-fluorescent structure of 3–4 μm in diameter was found that could only be seen in two or three adhering optical sections. The region was marked in the DIC image by a small body. From this and in comparison with the ultrastructural evidence (Fig. 1 b), it became apparent that this body represented the nucleolus, which lacked any fluorescence. From the circumference of the nucleus, small bodies were seen budding off in the direction of the cytoplasm (Fig. 3 b). Sometimes, cells were

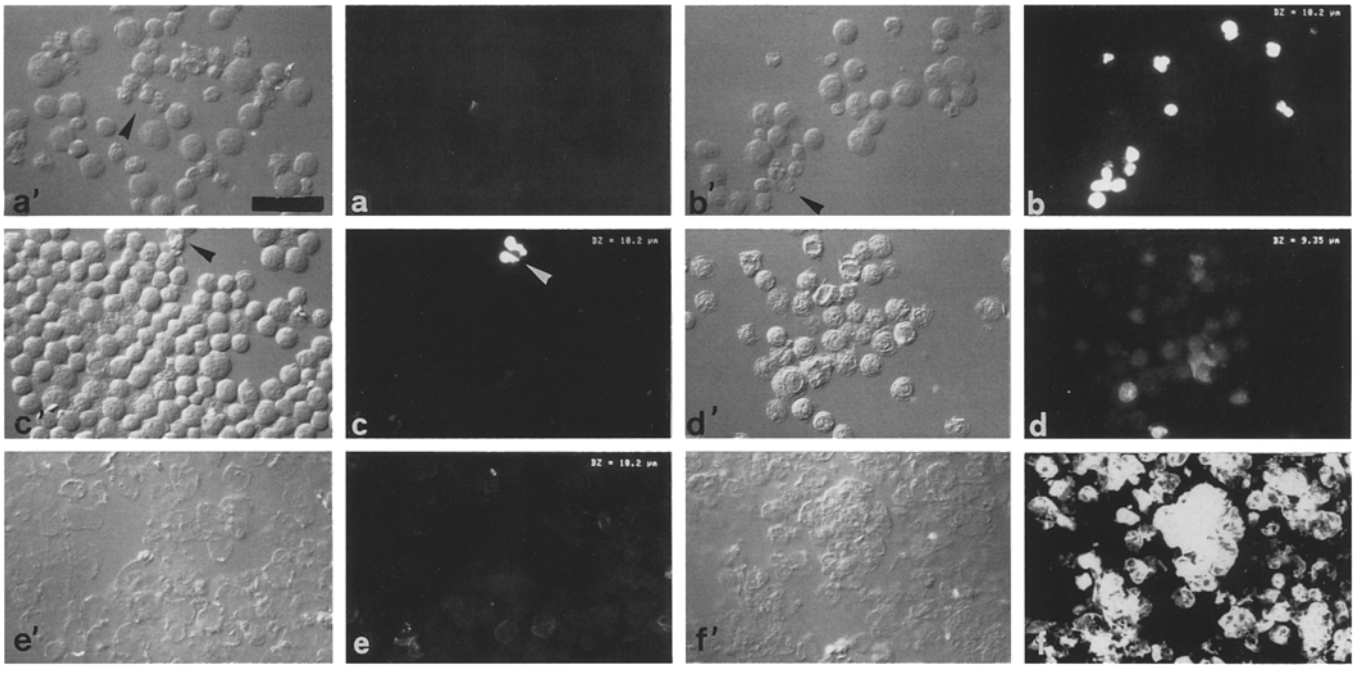

Fig. 4a–f, a′–f′. In situ 3′end labelling of necrotic cells. Fluorescence images **(a–f)** and corresponding interference contrast images **(a′–f′)**. Cultivation with ionomycin for 3 h **(a, a′)** and 24 h **(b, b′)**. The small shrunken cells (*arrowhead*) are killed by ionomycin. No labelling after 3 h, whereas strong labelling can be observed after 24 h. NaN_3 induced cell death after 3 h **(c, c′)**, and 24 h **(d, d′)**. No DNA fragmentation can be found after 3 h. *Arrowhead* points to a spontaneous apoptotic cell, as found in controls (compare Fig. 2 a, a′). Diffuse variable fluorescence appeared in some cells after 24 h. Necrosis after freezing and thawing: distinct cellular destruction, but no labelling after 3 h **(e, e′)**; intense fluorescence, not associated with cellular or nuclear margins, after 24 h **(f, f′)**. *Bar*: 56 μm

found whose nucleus had fragmented at an earlier stage into large bodies that were again outlined by a peripheral, intensely fluorescent crescent and diffuse staining over the entire body, whereas the cytoplasm itself remained unlabelled (Fig. 3 c). The second group, which represented about 90% of labelled cells, was characterised by the nucleus having disappeared and the cell being full of smaller nuclear fragments that varied in size from about 1–5 μm and that appeared by confocal laser microscopy to be homogeneously fluorescent (Fig. 3 d). Occasionally, a rare cell was found in which some of these bodies were half-moon shaped or had a central clear region (Fig. 3 e). Most cells in this group were also characterised by diffuse fluorescence in the cytoplasm (Fig. 3 d), whereas the cells in which cytoplasmic staining was absent generally had a more degenerated morphology in the DIC image. A third type of apoptotic nuclear alteration could be distinguished; this was however only infrequently observed. It consisted of a large homogeneously fluorescent nuclear body with only a few or no nuclear fragments inside the cytoplasm, and appeared as a nucleus with fragmented and condensed DNA but with no concomitant fragmentation into multiple bodies (Fig. 3 f). The frequency of the various morphological types of apoptotic cells was no different after 0, 3 and 24 h cultivation with actinomycin D, although the total number of apoptotic cells increased dramatically after prolonged cultivation (see Fig. 5 a).

Necrotic cell death

In order to investigate different pathways for necrotic cell death, necrosis was produced in three ways in order to compare the occurrence and appearance of DNA fragmentation with that in apoptotic cells. Ionomycin is a well known Ca^{++} ionophore that induces necrotic cell death as demonstrated by cytolysis in the absence of internucleosomal DNA fragmentation (Albritton et al. 1988; Ojcius et al. 1991). The morphology of the cell death occurring after exposure to ionomycin was studied ultrastructurally. As early as 3 h after treatment, dead cells were seen that were reduced in size and characterized by a disrupted plasmalemma, vesicular rough ER profiles and swollen electron-dense mitochondria. The nuclear envelope had dissapeared and the nucleus contained a finely dispersed matrix, but no heterochromatin and no nucleoli (Fig. 1 d).

To mimic the effect of acute ATP depletion, a common final pathway of necrotic cell death, cultures were exposed to NaN_3, which causes ATP depletion in cells (Lockwood 1988; Schmid and Carter 1990). Transmission electron microscopy revealed darkly stained cells with severely damaged membranes, the absence of cell

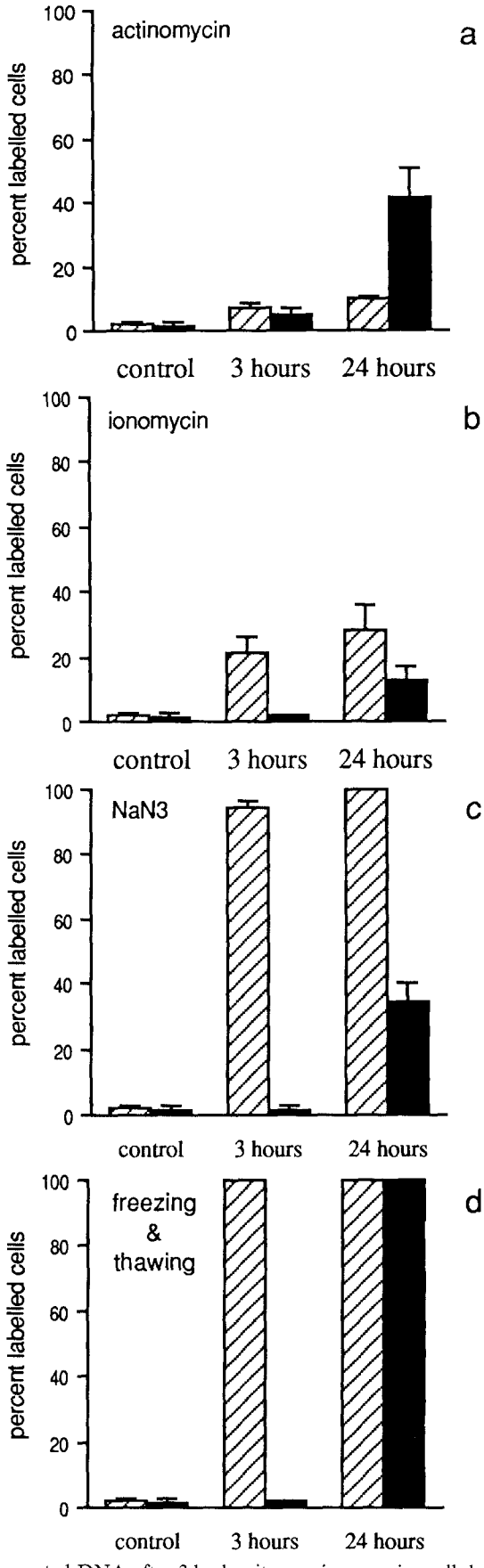

Fig. 5a–d. Mean percentage of stained cells (± SE) in the nigrosin dye exclusion test (*hatched columns*) and after in situ 3′end labelling (*black columns*). After induction of apoptosis by actinomycin D for 24 h, the number of cells with fragmented DNA is significantly increased and exceeds the number of dead cells as determined by the nigrosin dye exclusion test **(a)**. Necrotic cell death **(b, c, d)** on the other hand leads to no increase in the number of cells with fragmented DNA after 3 h, despite causing massive cell death, as indicated by the nigrosin dye exclusion test. After 24 h, the dead cells become positive for DNA fragmentation. Values represent the mean of three independent experiments and the standard error of the mean

organelles and a coarse chromatin that had spilled over into the cytoplasm (data not shown).

Rapid freezing in liquid N_2 and thawing in three cycles caused death of all cells by the disruption of cellular membranes. Necrosis was also evident ultrastructurally after this treatment; the nuclei were severely disrupted and no cytoplasmic organisation remained (data not shown).

In situ 3′end labelling of DNA breaks was carried out for all experiments involving necrosis. After 3 h, no increase of labelled cells could be observed (Fig. 4 a, c, e). The positive cells were similar in morphology to apoptotic cells and probably represented the spontaneous apoptotic cells seen in untreated cultures. The large majority of cells however proved to be dead by the nigrosin test, appeared severely damaged by DIC and showed no labelling. In sharp contrast, after 24 h, the dead cells displayed distinct fluorescence. The intensity and the morphological appearance was not uniform and varied widely among the three experimental groups (Fig. 4 b, d, f). The nucleus of cells killed by ionomycin was marked by an intense homogeneous fluorescence (Fig. 4 b). The labelling intensity in the NaN_3-exposed group was only faint and varied widely among individual cells (Fig. 4 d). Bright fluorescence was seen on slides with cells that had undergone rapid freezing and thawing 24 h previously. It corresponded to cytoplasmic and chromatin debris, as nuclear and cellular margins were no longer demarcated (Fig. 4 f).

In all groups showing necrotic cell death, the chromatin of an individual cell became evenly positive, with no local preponderance or recognizable substructures; the labelling had the same cellular localization as the disrupted nuclear chromatin after cell death. The orderly pattern of peripheral nuclear crescents and the fragmentation of the cell and nucleus into many bodies, as observed in apoptosis, could not be found in necrosis.

Quantitative analysis of cells stained by nigrosin and by 3′end labelling revealed a comparable number of labelled cells 3 h after culturing in the presence of actinomycin D (Fig. 5 a). After 24 h, more stained cells could be counted following 3′end labelling than by the nigrosin dye exclusion test, indicating the presence of a distinct percentage of cells with fragmented DNA but an intact plasma membrane. In contrast, in necrosis experiments a significant increase in the number of nigrosin-stained cells could be found reaching nearly 100% in NaN_3 and freezing plus thawing experiments after 3 h, whereas 3′end labelling revealed no rise of positive cells compared with controls (Fig. 5 b, c, d). However, after 24 h, a variable number of cells also became stained by in situ 3′end labelling. Thus, the necrosis experiments showed a clear uncoupling between membrane damage and DNA fragmentation that had apparently occurred several hours after the cells had died.

Discussion

We have been able to demonstrate the presence of DNA fragments in the nucleus and cytoplasm of cells by means of in situ 3′end labelling. Confocal laser microscopy has revealed the spatial arrangement of these fragments within the cell. Therefore the continuing DNA fragmentation during the process of apoptosis has been observed on at the single-cell level.

In cultures stimulated to undergo apoptotic cell death by actinomycin D treatment, which is known to induce apoptosis (Martin et al. 1990; Cotter et al. 1992; Inouye et al. 1992), brightly fluorescencing cells appear, whereas the remaining cells are completely negative. The clear distinction between negative and positive cells and the different stages of nuclear fragmentation observed demonstrate that apoptosis is an asynchronous event, in which some cells enter the apoptotic pathway at different time points, whereas the remaining cells are unaffected. A few apoptotic cells can always be found in untreated K562 cultures.

No transitional stages between cells with an unfragmented genome and cells with extensive DNA fragmentation have been found. Labelled cells, which are regarded as being in the initial stage of apoptosis on the basis of their nuclear morphology and extent of labelling, are intensely fluorescent around the nuclear circumference. This shows that the breakdown of the nuclear chromatin after endonuclease activation is a rapid event, probably beginning at multiple sites and directing the cell unequivocally to apoptotic cell death. This observation also corresponds well with results of density-gradient centrifugation, in which apoptotic cells can be clearly separated by their increased buoyant density from healthy cells, with no intervening cells in between (Arends et al. 1991; Cotter et al. 1990), demonstrating apoptosis as being a one-step procedure.

A comparison of the ultrastructure and in situ 3′end labelling of actinomycin-D-exposed cells reveals that many DNA breaks are present, probably as a result of the condensation of fragmented chromatin, in the electron-dense chromatin masses that are characteristic for apoptotic cell death. However, DNA fragments can also be found in areas that appear electron translucent. Whether these areas are stained by chromatin fragmentation in loco, or by mono- and oligonucleosomes that have been transferred into the interior of the nucleus after breakdown in the peripheral crescents remains unknown. Therefore questions regarding whether the endonuclease causing the DNA fragmentation is located outside the nucleus and after activation gains access to the interior, or whether the enzyme is already present in the nucleus and only needs to be activated remain to be answered by direct immunolocalisation of the enzyme. We favour the hypothesis that chromatin breakdown starts over the whole nucleus and that the fragmented chromatin accumulates into crescents at the nuclear periphery until all accessible DNA strands have been cleaved by the endonuclease. In this way, the nuclear chromatin becomes not only fragmented, but also more densely packed and reduced in volume. It is also unclear whether the action of the endogenous nuclease alone is sufficient for the nuclear changes, as suggested by Arends et al. (1990). Other factors might be involved in the peripheral chromatin condensation and nuclear fragmentation. In this respect, the observation that the budding of small nuclear fragments always occurs in the direction of the remaining

cytoplasm favours the hypothesis that other factors, such as cytoskeletal proteins, play a role.

DNA fragmentation in necrotic cells appears to be different. Onset is delayed, i.e. it does not occur at the time of cell death, indicating that it is a passive degenerative event and not an active process. Therefore no orderly regular pattern of DNA fragmentation can be found, although the chromatin is slowly digested. Moreover, the nuclear DNA appears to be remarkably resistant to cleavage, because necrotic cells usually show extensive nuclear destruction ultrastructurally, whereas at the same time, no detectable DNA fragmentation exists as revealed by in situ 3′end labelling. The structural changes of the chromatin may therefore be caused by precipitation or salting out in the absence of DNA breaks. The later occurring DNA fragmentation can best be interpreted as a result of hydrolysis by lysosomal enzymes that become liberated secondarily and that attack the nuclear chromatin, as suggested by Afanas'ev et al. (1986). However, the endogenous nuclease responsible for apoptosis may become unspecifically activated after necrotic cell death.

From our results, the demonstration of a fragmented genome by in situ 3′end labelling can only be considered specific for apoptosis if it is associated with the characteristic apoptotic morphology or if it occurs at the onset of cell death. Recently, in situ 3′end labelling by terminal transferase has been used for the identification of programmed cell death in histological sections (Gavrieli et al. 1992). However, for a definitive identification of apoptotic cell death in tissues, where necrosis cannot be excluded a priori, demonstration of DNA fragmentation alone is not sufficient, because necrotic cells in tissues also undergo progressive chromatin dissolution, a process described as karyolysis by pathologists.

In summary, our study confirms the concept of characterising cell death as either apoptosis or necrosis, which are triggered by different intracellular pathways and which are biologically different phenomena. Both types of cell death should be taken into consideration for experiments investigating cell damage in response to toxic or infectious agents, or hormonal signals. Usually, cell death is assessed by dye exclusion tests or the release of cytoplasmic markers, both of which reveal the membrane damage of dying cells, but not DNA fragmentation. In our study, the determination of cell death by actinomycin D after 24 h on the basis of dye exclusion tests alone would have resulted in a large underestimation of the real number of dead cells (Fig. 5 a). Thus, in situ 3′end labeling allows the quantification of the number of dying cells with fragmented DNA and provides structural information that may be valuable in further investigating the significance of cell death in unclear cases.

Acknowledgements. The authors wish to express their gratitude to Mrs. M. Erni for electron microscopy, to Mr. W. Scherle for technical assistance and to Mr. H. Sonderegger for photographic work.

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