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Variable Expression of Apoptotic Phenotype in Keratinocytes Treated with Ultraviolet Radiation, Ceramide, or Suspended in Semisolid Methylcellulose

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Apoptosis is a form of cellular suicide and is activated in various cells, including keratinocytes, in response to physiological and pathological stimuli. A current hypothesis holds that apoptotic cells in a concerted manner express characteristic phenotypic features comprising cytoplasmic budding, pyknosis, chromatin condensation, karyorhexis and internucleosomal DNA fragmentation. In this study we investigated the effects of different potential inducers of apoptosis on cultured human keratinocytes. Viability was determined with vital dyes (ethidium bromide, trypan blue), cell morphology was investigated with electron microscopy, and nuclear and DNA integrity was assessed with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) or DNA gel electrophoresis. Irradiation with 50 mJ/cm^2 ultraviolet B (UVB), treatment with C8 ceramide, or suspension in a semisolid medium caused apoptosis which was ultrastructurally different from necrotic induced by very high (300 mJ/cm^2) doses of UVB. However, the phenotype of dying cells did not exhibit all typical features of apoptosis and cell morphology depended on the method used to induce apoptosis. Cells irradiated with ultraviolet B or treated with C8 ceramide developed large and small budding and DNA nicks but not chromatin condensation or classical karyorhexis. In UVB-irradiated cells a novel form of karyorhexis was observed manifested by formation of a few very small chromatin fragments in nuclear periphery. Cells suspended in methylcellulose developed DNA nicks and pyknosis, but not budding or karyorhexis. In neither case could the typical internucleosomal DNA fragments be detected by gel electrophoresis. These morphological results indicate that in keratinocytes induction of effect or death mechanisms is not concerted but depends on the stimulus inducing apoptosis. ***Key words: adhesion; cell death; ceramides; differentiation; ultraviolet radiation.***

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Recent research into the mechanisms of cell death have led to a distinction between necrosis and apoptosis. Apoptosis has been conceptualized as a form of cellular suicide which is based on a genetic mechanism, whereas the term "necrosis" has been reserved for accidental cell death due to direct injury (1, 2).

Cells dying by apoptosis develop characteristic structural changes which have been studied in detail in several cell types, mainly lymphocytes, hepatocytes, and tumour cells. The earliest evidence of the onset of apoptosis is found in chromatin that condenses and becomes aggregated in the periphery in sharply demarcated electron-dense masses (referred to as half-moon, horse-shoe, or navicular forms) (1, 3). In the next step, the nuclear convolution is observed, followed by pyknosis, disruption of the nucleus (karyorhexis), and budding resulting in formation of apoptotic bodies (3). Biochemically, an enzymatic caspase pathway is triggered leading to an activation of endonuclease which in turn cleaves DNA between nucleosomes leading to a generation of DNA fragments of approximately 185 base pairs. Those specific events are absent in necrosis, which is caused by osmotic or mechanical cell disruption and is represented by cell and nucleus swelling, diffuse chromatin clumping, swelling of mitochondria, disappearance of ribosomes, surface blebbing rather than budding, and plasma membrane disruption. DNA is cleaved non-specifically, resulting in the formation of a smear rather than internucleosomal laddering in electrophoresis.

According to prevalent opinion, the common apoptotic phenotype is encountered in most cells independently of the tissue of origin, or the type of stimulus inducing apoptosis. For example, lymphocytes treated with such unrelated factors as ultraviolet radiation (UV), glucocorticoids, cytostatics, or ionizing radiation develop the same sequence of morphological changes encompassing internucleosomal DNA fragmentation, chromatin condensation, pyknosis, cytoplasm budding, and karyorhexis (4–6). Deviations from this common pattern are possible, such as lack of internucleosomal DNA fragmentation or chromatin condensation in some cells (7–9) but have been considered as exceptions rather than the rule.

In this paper we compared morphological and biochemical features of cultured keratinocytes triggered to apoptosis by different stimuli: UVB radiation, membrane-permeable ceramide C8, and suspension in semi-solid methylcellulose. Apoptosis induced by UVB has been widely studied in keratinocytes from different species (10–13). Cell death is probably triggered by DNA damage and is mediated by p53 protein (10, 14). Ceramides belong to another class of apoptosis-inducing factors which trigger cell death independently of DNA damage. Ceramides generated intracellularly by sphingomyelin hydrolysis are probably the major mediators of apoptosis induced by several cytostatic drugs, such as daunorubicine, or Fas-induced cytotoxicity (6, 15). In epithelial cells, apoptosis may also be triggered by a loss of adhesion (so-called anoikis) (16). Anoikis, which is preceded by terminal cell differentiation, may be induced in keratinocytes suspended in the methylcellulose semi-solid medium (17, 18).

We show here that morphology of apoptotic keratinocytes depends on the type of stimulus used to induce cell death.

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MATERIAL AND METHODS

Keratinocyte culture

Cryopreserved normal human keratinocytes were purchased from PromoCell (Heidelberg, Germany) and cultured in the keratinocyte growth medium, which consisted of the modified MCDB153 medium containing 0.09 mM $CaCl_2$, 50 μg/l recombinant human EGF and 0.1% bovine pituitary extract (Gibco Life Technologies, Gaithersburg, MD). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and passaged at 80–90% confluence after trypsinization with 0.2 ml/cm^2 0.05% trypsin with 0.02% EDTA solution. Third passage cells were used for experiments.

Cell treatment

For UV irradiation the media were changed to phosphate-buffered saline (PBS). The cells were irradiated from above with a Philips TL12 lamp at a distance of 35 cm, as previously described (10). The TL12 emits broad-spectrum UVB radiation and the detailed emission spectrum has been described in detail elsewhere (19). Controls were sham-irradiated with blue light under the same conditions and for the same periods of time as the experimental UV-irradiated cells. After irradiation the cells were incubated in the standard medium.

A cell membrane permeable *N*-octanoyl-*D-erythro*-sphingosine (C8 ceramide) was purchased from Calbiochem (Cambridge, MA) and solubilized in DMSO. The compound was added to the cultures at final 10 μM concentration. The final concentration of DMSO was 0.1%.

For suspension in methylcellulose, keratinocytes were trypsinized at 37°C with 0.05% trypsin with 0.02% EDTA and suspended at density 10^6 cells/ml for 24–48 h in the semi-solid methylcellulose as described previously (20). After incubation, cells were diluted and washed twice with the keratinocyte growth medium and twice with PBS.

For treatment in high calcium concentrations, the concentration of this ion was adjusted to 1.8 mM by adding the appropriate volume of the stock 100 mM solution of $CaCl_2$ in water.

Survival assays and fluorescence microscopy

Two vital dyes were employed to investigate cell viability, ethidium bromide (EB) and trypan blue, as described in previous studies (21, 22). EB (Sigma, St. Louis, MO) was used at final 2 μM and the cells were loaded for 15 min at room temperature. This dye stains nucleic acids in non-viable cells. For the trypan blue exclusion assay, the cells were incubated for 5 min with 0.1% solution of the dye (Sigma), trypsinized and counted manually in Bürker cammer. Nuclei were visualized by staining with 1 μg/ml 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim). The cytochemical detection of internucleosomal DNA fragmentation *in situ* was performed with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method as described by Gavrieli et al. (23) using an In Situ Cell Death Detection Kit (Boehringer Mannheim). The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis and discriminates the strand breaks induced by radiation or cytostatic drugs from those seen in apoptosis (5, 24). Labelling was performed as suggested by the manufacturer. Briefly, the cells were washed once in PBS, fixed at –20°C in a 1/1 (v/v) mixture of methanol and acetone, rehydrated and blocked with 1% bovine serum albumin (fraction V, Sigma, St. Louis, MA). The 3′-OH termini of internucleosomal DNA strand breaks were labelled with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase. Control experiments showed negative staining of sham-, blue light-irradiated keratinocytes and absent nuclear staining in samples in which the terminal deoxynucleotidyl transferase was omitted. EB or DAPI stained cells were investigated with Olympus BX70 microscope. TUNEL stained samples were observed in a confocal, laser-scanning microscope (TCS4d, Leica Laser Technik GmbH, Heidelberg, Germany). One recording consisted of a series of 5–20 optical sections, from the top to the bottom of the sample. To determine proportions of labelled cells, a minimum of 100 cells were counted. Data are expressed as means with standard deviations (SD), or 95% confidence intervals (95% CI).

Electron microscopy

Keratinocytes were grown on plastic Thermanox coverslips (Nunc, Roskilde, Denmark) in the standard medium until confluent cultures were obtained, and treated as required. Adherent cells were processed for transmission or scanning electron microscopy using standard protocols. In some instances, the cells were cultured in 80 cm^2 plastic flasks, trypsinized, pelleted, and prepared for transmission microscopy.

DNA electrophoresis

Keratinocytes were removed from plastic with a rubber policeman. DNA was extracted as described previously (25) and resolved on 2% agarose gel. The gels were stained with ethidium bromide and photographed under UV light.

RESULTS

UVB-induced keratinocyte death

When subconfluent keratinocyte cultures were irradiated with 50 mJ/cm^2 UVB, EB labelling of cells occurred 12 h after irradiation and increased significantly during next 48 h when it reached its maximum (Fig. 1). The same shape of a curve was found when the cells were labelled with trypan blue (not shown). However, when TUNEL labelling was performed, the staining was delayed, peaking 72 h after irradiation.

To study nuclear morphology, DAPI-stained or TUNEL-labelled cells were analysed in fluorescent microscope. After irradiation, a variable degree of fluorescence after TUNEL staining was seen (Fig. 2). The staining was predominantly homogenous and classical karyorhexis (defined as a breakdown of nucleus into two or more fragments of comparable sizes) was very rarely encountered (0.1–0.5% cells in confluent cultures, 95% CI). However, many nuclei (19–36%) demonstrated a limited budding with emergence of a small globular fragment in the periphery (Fig. 2). This feature was only seen

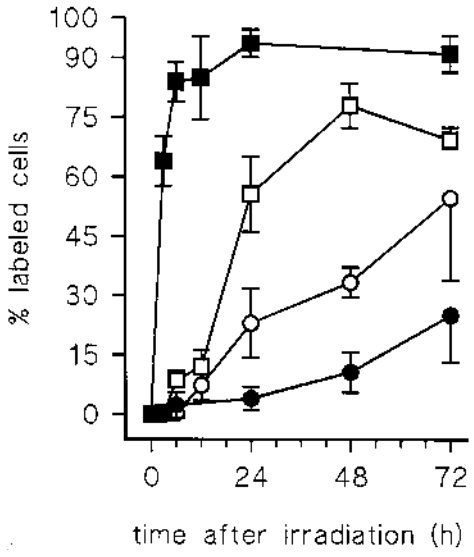
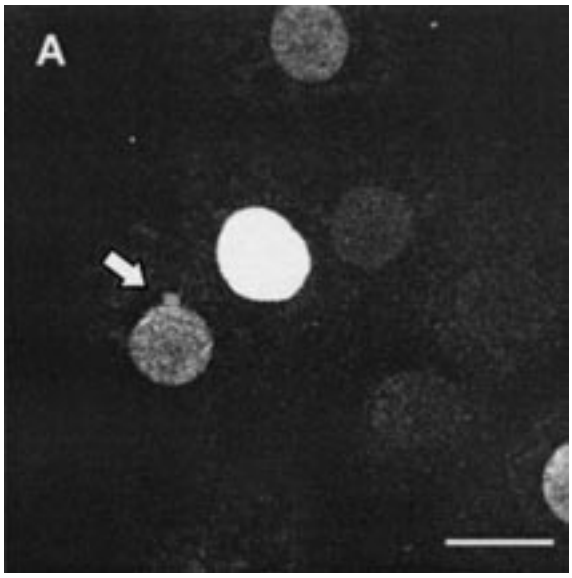
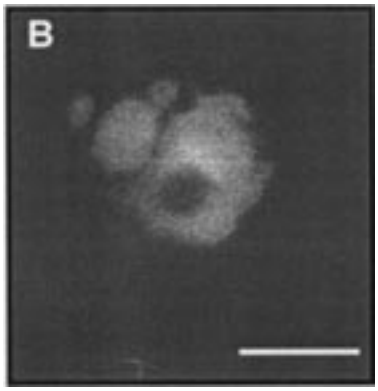
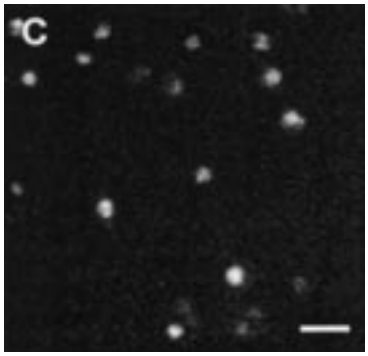


Fig. 1. Cell death induced by UVB in cultured human keratinocytes. The cells were cultured to near confluence and irradiated with 50 mJ/cm^2 (open symbols) or 300 mJ/cm^2 (closed symbols). At different times after irradiation the cells were labelled with ethidium bromide (squares), or TUNEL (circles). Symbols represent means ($n=3$) with SD.







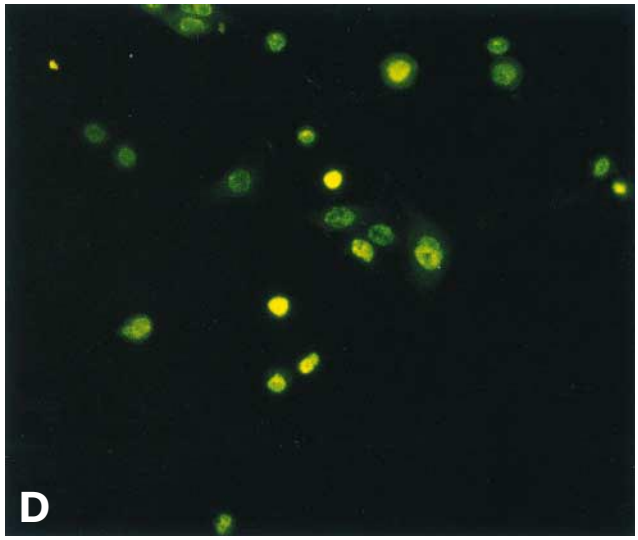


Fig. 2. TUNEL staining of UVB-irradiated keratinocytes. Cells were irradiated with 50 mJ/cm^2 UVB, stained with TUNEL after 24 h, and observed in laser scanning microscope (*A–C*) or fluorescence microscope (*D*). *A, B, D* show staining in adherent cells, staining of floating cell 24 h after irradiation is in *C*. Note different intensity in staining (*A, D*) and formation of globular nuclear fragments containing nicked DNA (*arrows* in *A*) and pyknosis (*C, D*). Karyorhexis was encountered only exceptionally (*B*). Bar: 10 μm in *A*, 5 μm in *B*, 20 μm in *C*.

in confocal microscopy on TUNEL-stained cells and was not apparent in DAPI-stained nuclei observed in fluorescence microscope. Only a minority (6–12%) of TUNEL-stained nuclei were pyknotic and a similar proportion of pyknotic cells was present in DAPI-stained cultures (Fig. 2).

To study the ultrastructure of UVB-irradiated keratinocytes, the cells were investigated with scanning and transmission electron microscopy. A striking feature observed in scanning microscopy was budding, which occurred rapidly (within 4–6 h) after irradiation with UVB (Fig. 3). Budding cells remained adherent for up to 72 h after irradiation. There were two types of buds: small buds similar to those described previously for apoptotic lymphocytes and cancer cells (26) and single large buds. Cells presenting smaller buds were most often encountered and sometimes large and small buds coexisted in one cell. In transmission electron microscope, irradiated keratinocytes presented characteristic budding (Fig. 4). The buds were filled with homogenous cytoplasm and were devoid of organelles or cytoskeleton elements. No defects in plasma membrane were seen. Mitochondria were normal but intermediate elements were often dispersed. Clear vesicles, which were occasionally encountered in normal cells, were more prominent and numerous in UVB-irradiated cells and localized mainly in the perinuclear area (Fig. 4). The nucleus of UVB-irradiated cells did not show any typical features of apoptosis (absence of chromatin condensation or marginalization). DNA electrophoresis of irradiated cells did not show evidence of DNA fragmentation during the first 24 h after irradiation, but in later periods a smear pattern was seen in the gels (Fig. 5).

To ensure that observed effects of UVB represented apoptosis rather than necrosis, the keratinocytes were irradiated with very high doses of UVB, which are known to cause a direct cell injury. Irradiation with 300 mJ/cm^2 caused a very rapid detachment of the cells and an increase in the proportion of EB labelled cells. TUNEL staining was suppressed in comparison to the keratinocytes irradiated with 50 mJ/cm^2 (Fig. 1). DNA smear was observed as soon as 24 h after irradiation (Fig. 5). Classical karyorhexis was encountered more often than in the cells irradiated with lower UVB doses (15–30% cells in confluent cultures; 95% CI). Ultrastructurally, irradiated cells had electron-lucent cytoplasm, granular chromatin, swol-

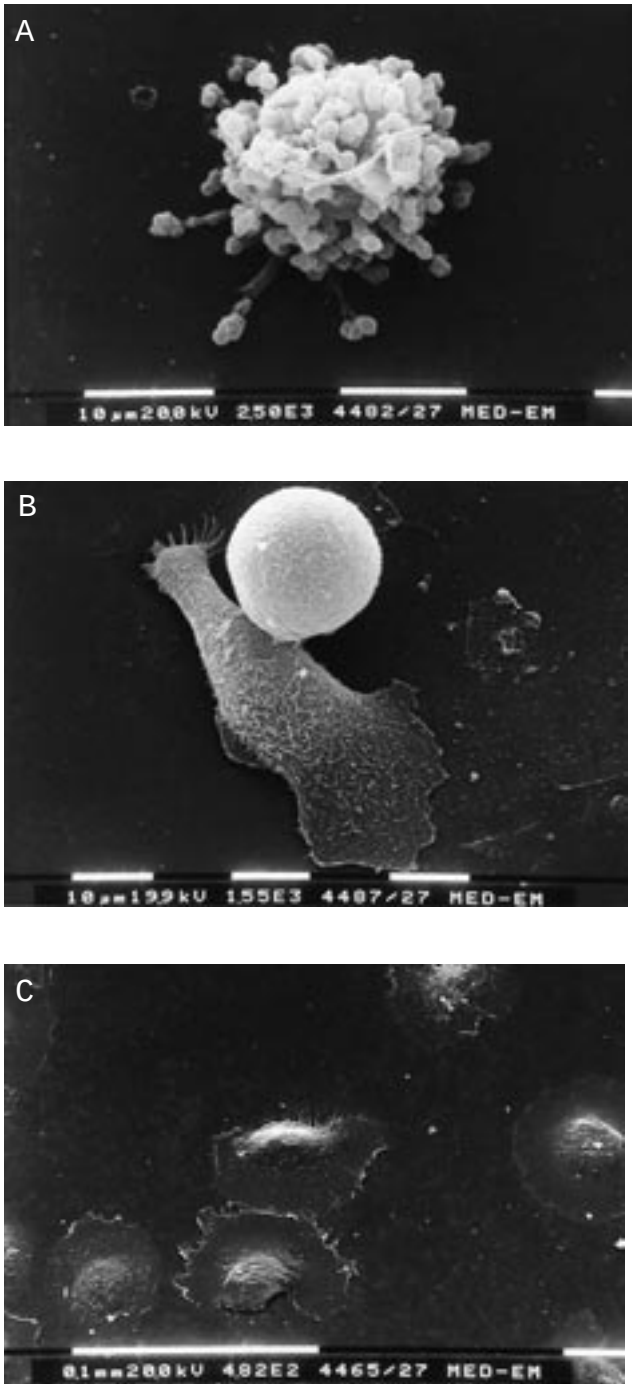


Fig. 3. Budding of UVB-irradiated keratinocytes. Cells were irradiated with 50 mJ/cm^2, incubated for 24 h, fixed and observed with scanning electron microscope. Two types of budding were observed: formation of small buds (*A*) and development of single large buds (*B*). Control, sham-irradiated keratinocyte is shown in (*C*). Bars: 10 µm in *A* and *B*, 100 µm in *C*.

len mitochondria and disrupted plasma membrane (Fig. 4), which agreed with known features of necrosis.

The above studies were done with the adherent population of keratinocytes. However, after irradiation with UVB a small proportion (6.2 ± 3.1% (SD) after 24 h; 8.7 ± 4.3% after 48 h) of cells detached from plastic and floated in culture media. Only a negligible proportion of floaters was present in control, sham-irradiated cultures (< 0.5%). As judged by staining with trypan blue and EB, 96.6 ± 2.1% (SD) of detached cells in UV-irradiated cultures were not viable. Staining with TUNEL and EB revealed that nuclei of detached cells were condensed, pyknotic and contained DNA nicks (Fig. 2). In electron microscopy, the cells demonstrated budding and pyknosis without chromatin marginalization (Fig. 4). Thus, the main difference between adherent and detached cells was the presence of pyknosis and condensation in the latter. When detached cells were sampled 72 h after irradiation an increased proportion of necrotic cells was seen, the morphology of which resembled that of adherent necrotic cells shown in Fig. 4*C*.

Cell death induced by loss of adhesion

Because of morphological differences between UVB-irradiated adherent and detached cells we hypothesized that the loss of contact with the substrate rather than the direct effect of UVB was responsible for observed changes in the nucleus. We therefore investigated morphological alterations in keratinocytes detached from the substrate (Fig. 6). The cells were trypsinized and suspended in a semi-solid methylcellulose medium. Detaching from the substrate, caused cell death as assayed by staining with EB (16.5 ± 5.2% (SD) after 24 h, 34.1 ± 8.9% after 48 h). Nuclei of detached cells remained condensed and pyknotic and labelled strongly with TUNEL. There was no evidence of internucleosomal DNA fragmentation (Fig. 6). In electron microscopy, the cells were small, condensed with pyknotic nuclei but without evidence of budding . After prolonged incubation (> 72 h), detached cells showed necrotic changes similar to those observed in detached, UVB-irradiated cells (not shown).

Effects of incubation in media containing high calcium concentrations

Previous studies have suggested that elevation of intracellular calcium concentration is the main mechanism mediating the development of pyknosis and chromatin condensation (27), but has little significance in DNA fragmentation. Treatment of keratinocytes with high calcium ion concentration leads to a persistent increase in cytoplasmic calcium concentration and the terminal differentiation of keratinocytes (28). When keratinocytes were incubated for 24 h in media containing 1.8 mM calcium ion concentration, pyknosis and chromatin condensation were seen focally after DAPI staining (Fig. 7). Single keratinocytes in calcium-high media became rounded and in some areas several keratinocytes adhered strongly to each other forming syncytial-like structures reflecting the process of differentiation (Fig. 7). Calcium-treated cells did not stain with TUNEL, nor did they develop other features of apoptosis, such as budding (Fig. 7), chromatin marginalization, or internucleosomal DNA fragmentation (not shown). Calcium-treated cells were viable and therefore could not be classified as apoptotic.

Cell death induced by ceramide C8

To investigate whether ceramides induced apoptosis in keratinocytes we treated subconfluent keratinocyte cultures with a membrane-permeable ceramide analog C8. EB and TUNEL

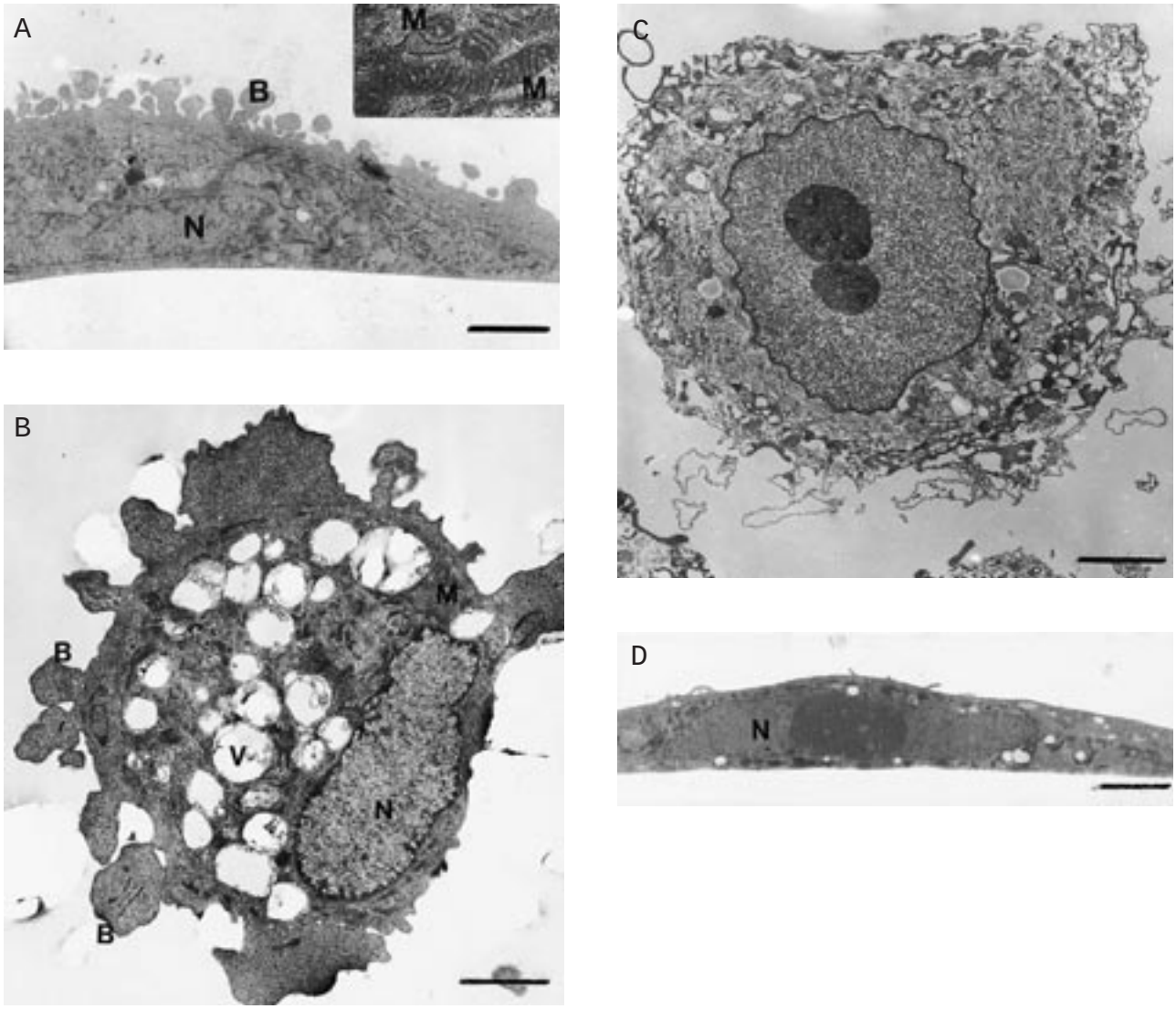


Fig. 4. Ultrastructure of UVB-irradiated keratinocytes. Cells were irradiated with 50 mJ/cm^2 (*A, B*), 300 mJ/cm^2 (*C*), or sham-irradiated (*D*), and photographed 24 h after irradiation. *A* and *D* show morphology of adherent cells; *B* and *C* show cell which detached 24 h after irradiation with 50 mJ/cm^2 and 300 mJ/cm^2, respectively. Insert in *A* shows mitochondrial ultrastructure. Cells irradiated with 50 mJ/cm^2 demonstrated prominent budding (marked with B), but their nuclei (N), chromatin, and mitochondria (M) were largely normal. 300 mJ/cm^2 induced typical features of necrosis (*C*). Note pyknosis in the detached cell in *B*, in comparison to nuclei of necrotic cells in *C* and normal suspended cells in Fig. 6B. Intracytoplasmic electron-lucent vesicles occasionally encountered in keratinocytes are marked with V. Bars: 0.5 μm in *A* (magnification $\times$3000, insert $\times$10 000), 1 μm in *B* ($\times$8000), and 2 μm in *C* ($\times$4000), *D* ($\times$3000).

stained the same proportion of keratinocytes (9–16%, 95% CI) but labelled nuclei only occasionally were condensed and pyknotic (Fig. 8). C8 ceramide induced large and small budding of adherent keratinocytes (Fig. 8). Transmission electron microscopy studies confirmed the presence of budding and lack of major alterations in the chromatin (Fig. 8). DNA electrophoresis showed unfragmented DNA up to 48 h after ceramide treatment, but 72 h after treatment a smear pattern was occasionally observed (Fig. 8).

DISCUSSION

Recent research has revealed that different morphological features of apoptosis are mediated by separate biochemical events, e.g. nuclear pyknosis is a calcium-dependent phenomenon and does not require endonuclease activation, an enzyme mediating internucleosomal DNA fragmentation (8, 27). With few exceptions (7, 9), most cells activated those biochemical pathways in a concerted mode, which leads to the development of a full apoptotic phenotype. The main finding of the present study was that in keratinocytes the type of apoptotic stimulus is an important determinant of the final phenotype. The results show unequivocally that keratinocytes possess the biochemical machinery necessary to produce pyknosis, chromatin condensation, karyorhexis and budding. Apoptosis could be induced by UVB, ceramide C8 or by suspending in semi-solid methylcellulose, but different phenotypes of apoptotic keratinocytes were observed in those cases (summarized in Table I). Apoptosis elicited by UVB or ceramide C8 was characterized by the presence of cytoplasmic budding and TUNEL staining of the

Table I. *Summary of morphologic changes in apoptotic keratinocytes treated with UVB, ceramide C8, or suspended in semisolid medium*[1]

Feature	UVB	Ceramide C8	Semisolid methylcellulose
Pyknosis and chromatin condensation	±	±	+
Chromatin marginalization	–	–	–
TUNEL staining	+	+	+
Karyorhexis[2]	+	–	–
Budding[3]	+	+	–
Internucleosomal DNA fragmentation	–	–	–

[1] –, Absent, ± present in a minority of cells, + constantly present.
[2] Predominantly non-classical karyorhexis (see Results).
[3] Both small and large budding.

nuclei, but pyknosis, karyorhexis and chromatin condensation was observed only in a very small proportion of apoptotic, TUNEL-stained, cells. In contrast, pyknosis and chromatin condensation were predominant features of apoptosis in the methylcellulose-suspended cells. However, pyknosis and chromatin condensation were present in UVB-irradiated or C8-ceramide treated keratinocytes which detached from the substrate and floated in the medium. This suggests that nuclear changes were precipitated by the loss of adhesion which occurred secondarily to UVB or ceramide exposure in a mechanism identical or similar to that seen in methylcellulose-suspended cells. It must also be noted that nuclear pyknosis was observed in differentiating keratinocytes exposed to high concentrations of calcium. Differentiation but not apoptosis took place in those cells. Cellular differentiation takes also place in suspended keratinocytes (17) and it has been argued that in both cases the mechanism of differentiation depends on the increase in the cytoplasmic calcium concentration (29). It is thus likely that elevation of intracellular-free calcium ion concentration is responsible for the development of pyknosis in keratinocytes, a conclusion which is in concordance with previous studies on isolated mouse liver nuclei (27).

Surprisingly, we were unable to detect such characteristic hallmarks of apoptosis as internucleosomal DNA fragmentation or marginalization of chromatin. Although with variable success, those features have previously been demonstrated in UV-irradiated human (11, 30), rodent (12, 13) or transformed (31–33) keratinocytes or in methylcellulose-suspended human keratinocytes (18). DNA electrophoresis performed in this study revealed that DNA was grossly intact in early phases of cell death. Random fragmentation producing a smear in gel electrophoresis and probably responsible for the positive TUNEL staining was seen in later phases of cell death and might be a result of a non-specific DNA degradation (3). Other authors were also unable to detect early DNA fragmentation. Hepatocytes treated with TGFß1 demonstrate all apoptosis hallmarks except internucleosomal DNA fragmentation (9, 34). Likewise, oligosomal DNA fragmentation, which is a common early event during cell death induced by cytotoxic T cells in lymphoid target cells, could not be demonstrated for fibroblasts and epithelial target cells, which tended to produce a smear pattern in DNA gel electrophoresis (35). Thus, internucleosomal DNA fragmentation is not a constant feature of

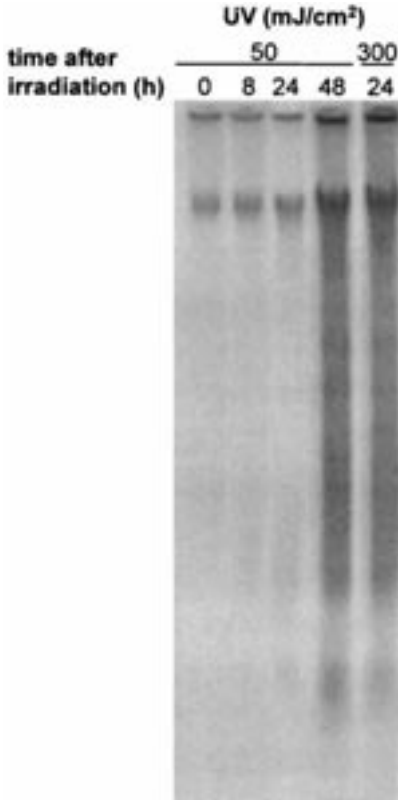


Fig. 5. DNA fragmentation after UVB irradiation of keratinocytes. Cells were irradiated with 50 mJ/cm^2 or 300 mJ/cm^2, and DNA electrophoresis in 2% agarose gel was performed at indicated times after irradiation.

apoptosis in keratinocytes. It is possible that activation of the endonuclease mediating internucleosomal DNA fragmentation depends on the composition of culture media and culture conditions. For example, Benassi et al. (11), who documented internucleosomal DNA fragmentation in human neonatal keratinocytes irradiated with UVB, treated with calcitriol, or cultured with high calcium concentrations, employed media containing suboptimal growth factor concentrations. In another study, where fully supplemented media were used, DNA laddering was much less conspicuous, resembling present electrophoresis results. It is well known that growth factors modulate apoptosis in keratinocytes (18). Modulation of the apoptotic process by growth factors will be the subject of future studies in this laboratory.

Some researchers use internucleosomal DNA fragmentation to distinguish between apoptosis and necrosis (1, 4, 36). This study shows that this approach may not be appropriate for keratinocytes. Even in the absence of DNA laddering it was obvious that observed changes represented apoptosis rather than necrosis. The majority of dying cells developed surface buds, possessed electron-dense cytoplasm with electron-lucent intracellular vesicles, morphologically normal mitochondria, and intact plasma membrane. In contrast, necrotic cells induced by very high doses of UVB were readily distinguishable by having electron-lucent cytoplasm with swollen mitochondria, ruptured cell membrane, and chromatin containing coarse-granular material. Moreover, there were differences in kinetics between apoptosis and necrosis. As judged by EB staining, necrosis developed during the first 24 h after irradia-

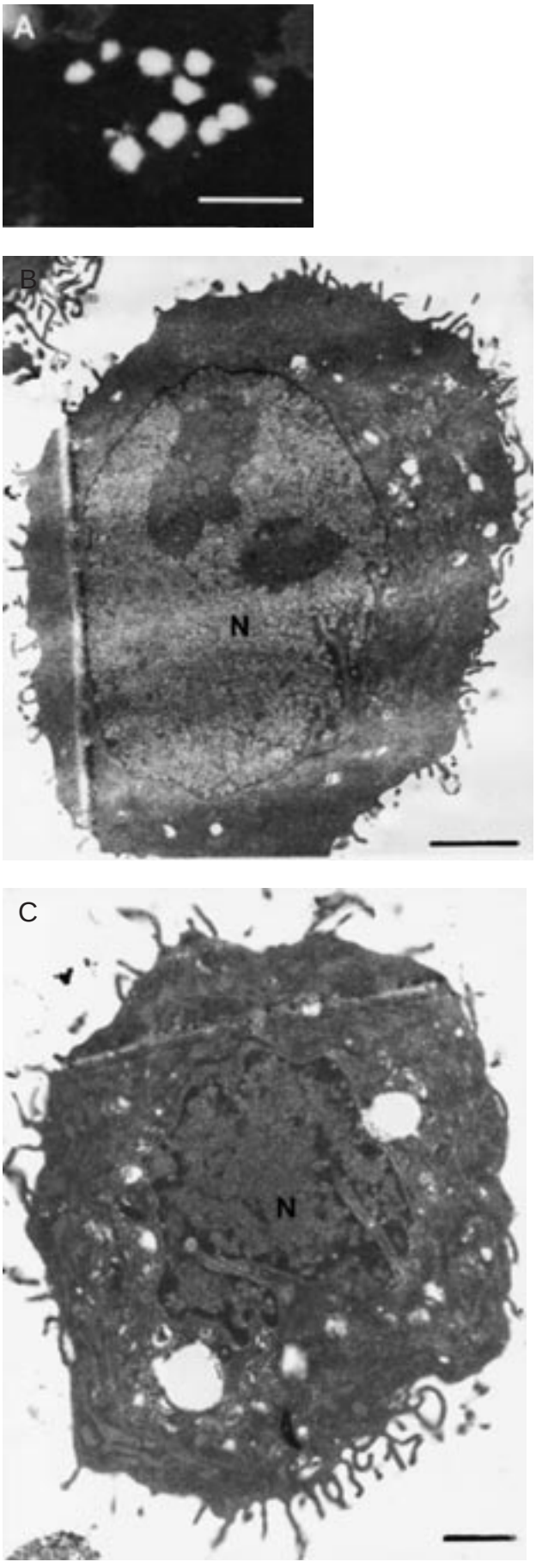


Fig. 6. Apoptosis in keratinocytes suspended in the semi-solid medium. The cells were trypsinized and suspended for 24–72 h in methylcellulose. *A* shows TUNEL staining at 48 h. Ultrastructure of control trypsinized cells and cells suspended for 48 h is shown in *B* and *C*, respectively (N-nucleus). Note pyknosis and absence of budding in *C*. Bars, 15 μm in *A*, 2 μm in *B* (magnification ×6000), and 1 μm in *C* (×6000). *D*-DNA electrophoresis of control cells and 24–48 h in suspension.

tion, whereas apoptotic cells accumulated during a 48 h period after UVB exposure.

Observations of karyorhexis and cytoplasmic budding revealed novel, previously undescribed features. Typically, karyorhexis is a breakdown of a nucleus to several smaller parts of comparable size. This form was only exceptionally seen in keratinocytes but was more often encountered in necrotic cells irradiated with very large UVB doses. However, confocal microscopy revealed an interesting variant of karyorhexis in keratinocytes irradiated with moderate doses of UVB, presenting as budding of a very small portion of a nucleus. This form of karyorhexis has not been seen after ceramide C8 treatment or suspension in the semi-solid medium.

Cytoplasmic budding has been considered a feature characteristic for apoptosis, in contrast to surface blebbing, which is also seen in necrosis (1, 3). The major difference between blebbing and budding is that in the latter case the cytoplasmic pouches are larger and do not result in formation of membrane defects. We observed here that, in addition to classic budding, keratinocytes are also able to produce very large, single buds. This feature is probably characteristic for apoptosis, since it

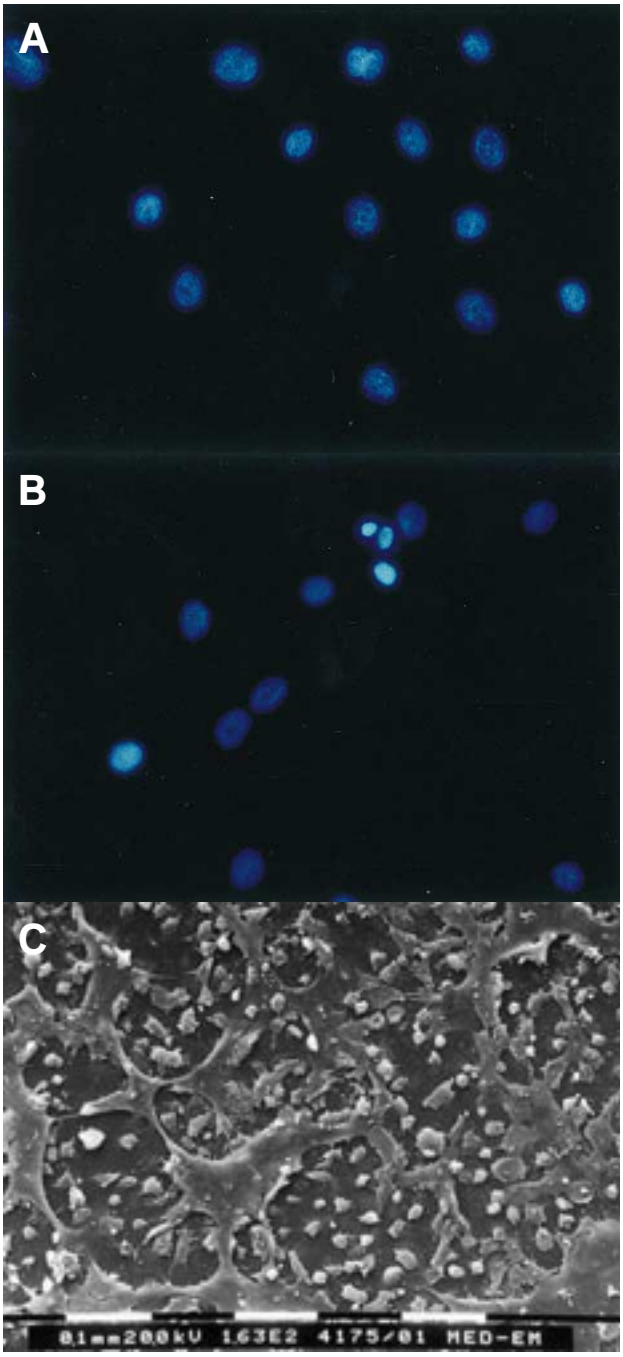


Fig. 7. Effects of calcium on cellular and nuclear morphology. Keratinocytes were incubated in medium containing 0.09 mM Ca^{2+} (*A*), 1.8 mM Ca^{2+} (*B–C*) for 24 h and stained with DAPI (*A, B*) or observed in scanning electron microscope (*C*). Scanning electron microscopy of control cells cultured with 0.09 mM Ca^{2+} is shown in Fig. 3*C*. Note nuclear pyknosis in *B* and absence of budding in *D*. Bar, 100 μm in *C*.

was observed after two unrelated treatments, UVB irradiation and incubation with ceramide C8.

Our observations in cell culture may help us understand the features of apoptosis *in vivo*. In the epidermis, apoptosis is a final pattern of the terminal keratinocyte differentiation (so-called diffapoptosis), or may be induced by different factors, such as UV radiation. Terminally differentiated, apoptotic keratinocytes stain with TUNEL and are localized in the transitional zone beneath stratum corneum (23, 37). Comprehensive ultrastructural studies did not reveal karyorhexis, budding, or chromatin marginalization in these cells (38, 39). Thus, apoptotic cells in the transitional zone do not demonstrate all apoptotic features, resembling cultured keratinocytes triggered to diffapoptosis by suspending in semi-solid methylcellulose. Moreover, early apoptotic keratinocytes in stratum granulosum and upper stratum spinosum which can be labelled by TUNEL do not present nuclear condensation [unpublished observations and ref. (39)] indicating that this feature occurs late in the process of diffapoptosis.

UV-induced apoptotic keratinocytes in the epidermis can be identified by TUNEL staining and are also called sunburn cells (40). The dynamics of the development of morphologic changes in irradiated keratinocytes in the epidermis resembles that reported here *in vitro*. Ultrastructural alterations (cytoplasmic vacuole formation) develops relatively early (5 h after irradiation), but other alterations expected for apoptosis (pyknosis, karyorhexis, budding) are not observed (41). Pyknosis and dyskeratosis develops 48–72 h after irradiation and are correlated with disruption of desmosomes (42). It is therefore likely that these changes represent late changes secondary to the loss of attachment, as seen in our *in vitro* model. A similar phenomenon has been observed in enterocytes, where expression of a mutant form of N-cadherin leading to disruption of cell–cell and cell–matrix contacts produces precocious entry into a death program (43). The fact that sunburn cells are encountered only in the upper epidermal strata supports the notion that a gap exists between the onset of apoptosis and development of pyknosis, which allows for migration from the basal layer to the more superficial layers of the epidermis. It should be underlined, however, that the doses of UVB required to induce apoptosis in cell culture exceeded those needed to cause formation of sunburn cells. It is likely that secondary mediators released due to UV treatment [such as tumour necrosis factor, ref. (32)] in the epidermis facilitate keratinocyte apoptosis.

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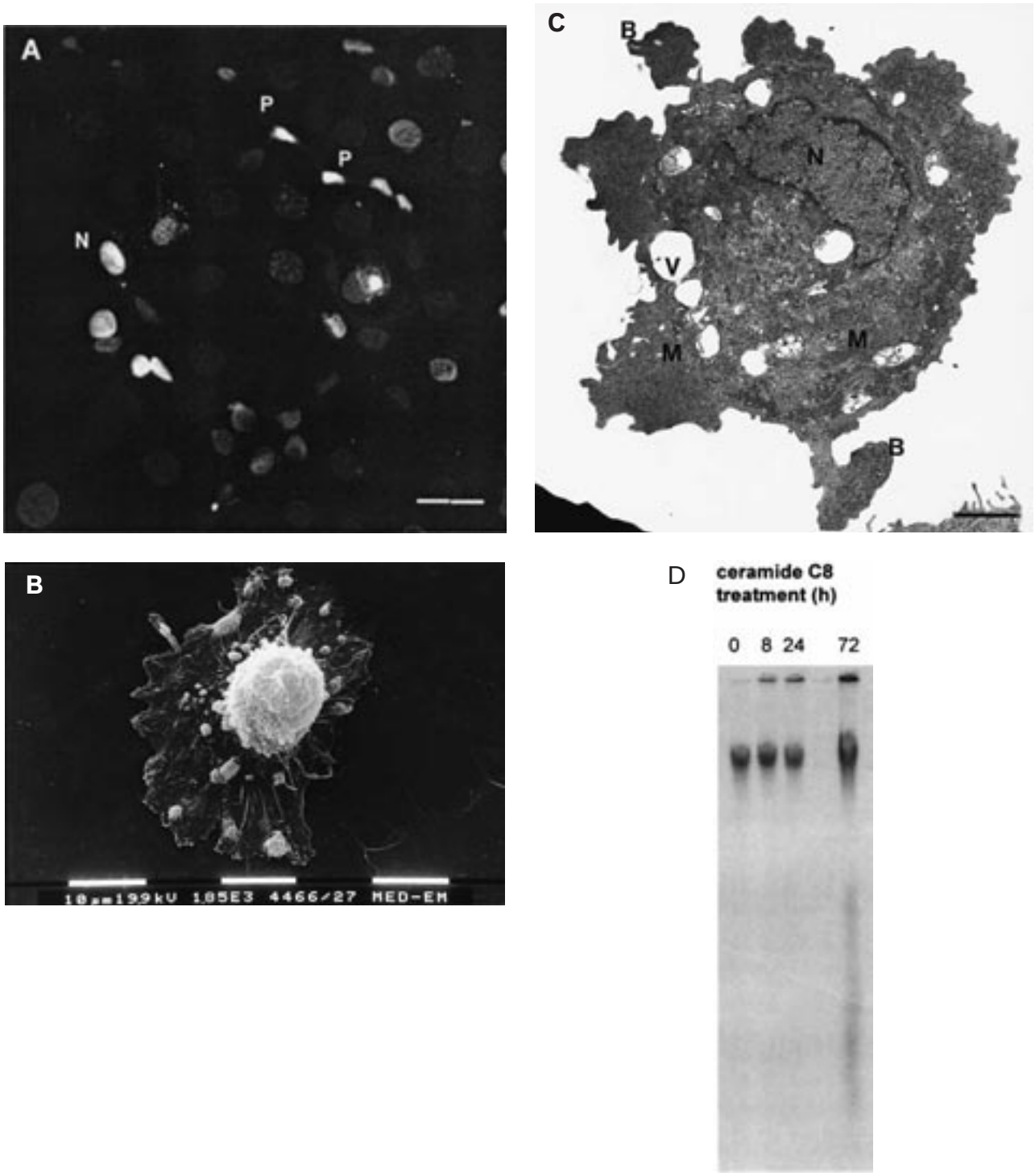

Fig. 8. Apoptosis of keratinocytes treated with C8 ceramide. The cells were treated with C8 ceramide for 24 h, fixed and stained with TUNEL (*A*) or processed for transmission electron microscopy (*B*) or scanning electron microscopy (*C*). Note that in *A* some stained nuclei have normal size (N), whereas some are clearly pyknotic (P). In *B*, budding (B), pyknosis of the nucleus (N) and preserved mitochondrial (M) structure are shown. *C* shows a cell demonstrating both large and small buds. Bars, 20 μm (*A*), 2 μm (*B*), 10 μm (*C*). Original magnification of *B* ×6000. As shown in *D*, integrity of DNA was preserved for 24 h; however, some degree of DNA fragmentation was encountered in cells treated with C8 ceramide for 72 h.

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