Apoptosis and Accidental Cell Death in Cultured Human Keratinocytes after Thermal Injury

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The respective roles of apoptosis and accidental cell death after thermal injury were evaluated in normal human epidermal keratinocytes. By coupling the LIVE/DEAD fluorescence viability assay with the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method and ultrastructural morphology, these two processes could be distinguished. Cells were grown on glass coverslips with a microgrid pattern so that the results of several staining procedures performed sequentially could be visualized in the same cells after heating at temperatures of up to 72°C for 1 second. After exposure to temperatures of 58 to 59°C, cells died predominantly by apoptosis; viable cells became TUNEL positive, indicating degradation of DNA. After exposure to temperatures of 60 to 66°C, both TUNEL-positive viable cells and TUNEL-positive nonviable cells were observed, indicating that apoptosis and accidental cell death were occurring simultaneously. Cells died almost immediately after exposure to temperatures above 72°C, presumably from heat fixation. The fluorescent mitochondrial probe MitoTracker Orange indicated that cells undergoing apoptosis became TUNEL positive before loss of mitochondrial function. Nucleosomal fragmentation of DNA analyzed by enzyme-linked immunosorbent assay and gel electrophoresis occurred after exposure to temperatures of 58 to 59°C. The characteristic morphological findings of cells undergoing apoptosis, by transmission electron microscopy, included cellular shrinkage, cytoplasmic budding, and relatively intact mitochondria. Depending on temperature and time of exposure, normal human epidermal keratinocytes may die by apoptosis, accidental cell death, or heat fixation. (*Am J Pathol* 1998; 153:567-577)

Burns are the most common accidental injury in the United States, affecting more than 2 million individuals and leading to 6500 burn and fire-related deaths per

year.^{1,2} Understanding the responses of skin to thermal injury may lead to improved treatment for these injuries.

Considerable information exists about hyperthermic death of cells in the context of the use of hyperthermia to eliminate cancerous tissue by exposure to temperatures in the range of 40 to 47°C.³⁻⁶ But little is known about the consequences of exposing normal human cells to the short-duration high temperatures that occur in burn injuries. An older study by Moritz and Henriques⁷ estimated that the basal layer of human epidermal cells could survive a maximum exposure of about 65°C for 1 second. We previously showed that cultured human epidermal keratinocytes could not survive a 1-second exposure above 58°C, but responded by synthesizing heat shock protein 70 (hsp70) and the chemokine interleukin-8.⁸ The purpose of the present study was to determine whether burn injury simulated by short pulses of high temperature causes cell death by apoptosis or by accidental cell death (ACD).

Although the importance of apoptosis in regulating cellular homeostasis through control of cell death is undisputed, discriminating an apoptotic cell from one dying from ACD is not a simple task.⁹ Morphological features of apoptosis that are readily recognizable at the light microscopy level, such as nuclear fragmentation, are present for only a short time and only during the final stages of the process. Progression toward the execution stage does not occur synchronously in all cells, rendering detection by a particular biochemical assay insufficient. Although both apoptosis and ACD lead to degradation of nuclear DNA, only apoptosis is gene directed, requiring the active participation of the targeted cell. To the extent that apoptosis occurs after thermal injury, it may be a target for rescue and improvement of epithelial cell healing after burn. The potential benefit of rescuing still-viable cells from undergoing apoptosis requires methods to discriminate between these two forms of cell death.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method developed by Gavrieli et al¹⁰ can provide an early indication that DNA fragmentation is occurring, and it is often used for evaluating apoptosis both *in vivo* and *in vitro*. It is not specific for apoptosis, however, because cells undergoing ACD can also be labeled by this method.¹¹⁻¹⁶ Accurate dis-

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crimination between cell death due to apoptosis or to ACD requires information about cell viability.

In the present study, normal human epidermal keratinocytes (NHEKS) were observed after heating to temperatures of up to 72°C for 1 second. Cells were cultivated on glass coverslips with a microgrid pattern so that the same cells could be reexamined after several staining procedures during a 72-hour period. After performing the LIVE/DEAD fluorescence viability assay to determine whether a cell was alive and recording this result, the cells were fixed and the TUNEL assay was performed on the same cells. Apoptosis was then defined as DNA degradation occurring within viable cells; TUNEL-positive cells were considered to be in apoptosis if they were alive by the LIVE/DEAD assay. Some cells may undertake, but not complete, apoptosis because of downstream defects in gene expression that cause them to undergo ACD. This progression is probably true for only a small number of cells.

Because mitochondria appear to play a prominent role in apoptotic cell death, the relationship between thermal injury and the mitochondrial permeability transition, reported to be a regular feature of apoptosis,¹⁷⁻²³ was also studied. The mitochondrial transmembrane potential probe MitoTracker Orange was used.

In addition, we examined the ability of thermally injured NHEKS to synthesize hsp70 and p53. hsp70 is often synthesized as a protective response to thermal injury. The expression of p53, on the other hand, appears to play a pivotal role in apoptosis,^{24,25} although the mechanism is unknown.

Materials and Methods

Cell Culture

NHEKS were obtained from skin discarded after reduction mammoplasty or abdominoplasty surgery. All tissues were obtained under informed consent. The epidermis was removed by floating 1 × 2-cm strips of skin on 0.25% dispase (Boehringer Mannheim, Indianapolis, IN) for 3 to 12 hours at 4°C. The epidermis was peeled away and treated with 0.25% trypsin/0.01% ethylenediaminetetraacetic acid for 30 minutes at 37°C.²⁶ The resultant cell suspension was filtered through a 100 µm stainless steel mesh to remove large debris and then plated in serum-free defined keratinocyte growth medium (Life Technologies, Inc., Grand Island, NY).²⁷ When confluent, primary cultures were subcultivated with trypsin/ethylenediaminetetraacetic acid or frozen in 10% dimethylsulfoxide for later use. All media for isolation and culture of keratinocytes contained penicillin (100 units/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml) (Life Technologies).

Thermal Injury

NHEKS were plated at 5×10^3 cells per well onto 13 mm-round standard glass coverslips and Microgrid coverslips (CELLocate, 175 µm, Eppendorf Scientific, Mad-

ison, WI) in 24-well multiplates (Costar, Cambridge, MA). Glass coverslips were pretreated with Pronectin F (Pro-mega, Madison, WI) and were used for fluorescence microscopy. Nunc Thermanox coverslips (Niles, IL) used for electron microscopy were uncoated. When the cells became confluent, the coverslips were removed with forceps and dipped into a sterilized circulating water bath (Lauda, Lauda-Königshofen, Germany) containing 3 liters of HEPES-buffered (10 mmol/L) saline for 1 second at temperatures of 56 to 72°C and then immediately dipped into saline at room temperature. The coverslips were then placed into a 24-well multiplate containing fresh medium and returned to the incubator.

LIVE/DEAD Assay

Coverslips with adherent cells were stained with 4 µmol/L calcein AM and 2 µmol/L ethidium homodimer-1 in phosphate-buffered saline for 10 minutes at room temperature in the dark (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes, Eugene, OR). Calcein AM is a membrane-permeant fluorogenic esterase substrate that is hydrolyzed in live cells to yield cytoplasmic, green fluorescence. Membrane-impermeant ethidium homodimer-1 labels nucleic acids of membrane-compromised dead cells with red fluorescence. Cells were analyzed by fluorescence microscopy with an Olympus BH-2 microscope equipped with a mercury lamp and filters from Omega Optical (filter set XF53; Brattleboro, VT), which provided excitation at 405 and 577 nm and emission at 525 and 650 nm.

MitoTracker Orange

Coverslips with adherent cells were stained with MitoTracker Orange (Molecular Probes) which was prepared in dimethyl sulfoxide and then added to the cell culture medium at a final concentration of 1 µmol/L. After a 15 to 30-minute incubation, the cells were analyzed by fluorescence microscopy using excitation at 525 nm and emission at 565 nm with the XF101 filter set from Omega Optical. Cells were considered MitoTracker Orange positive if a bright punctate orange fluorescence of the mitochondria was observed and MitoTracker Orange negative if cells exhibited a diffuse orange cytoplasmic staining.

TUNEL Assay

Coverslips with adherent cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. DNA fragments were labeled with the *In Situ* Cell Death Detection Kit, Fluorescein (Boehringer Mannheim). The kit was used according to the manufacturer's instructions, with the addition of incubation in citrate buffer for 30 minutes at 60°C before TUNEL reaction.²⁸ The coverslips were then incubated with anti-fluorescein-alkaline phosphatase (Boehringer Mannheim; 7.5 U/ml) for 45 minutes at 37°C in a humidified chamber, rinsed in phosphate-buffered saline, and incubated with Fast Red TR/Naphthol

AS-MX (Sigma Chemical Co, St. Louis, MO) for 10 minutes. Cells were counterstained with hematoxylin, mounted cell side down on a microscope slide, and analyzed by bright-field microscopy. TUNEL-positive cells appeared red, whereas TUNEL-negative nuclei appeared blue.

Image Acquisition

Fluorescence images were obtained with a VI-470 charge-coupled device video camera system (Optronics Engineering, Goleta, CA), and bright-field microscopic images were obtained with a Leaf Lumina charge-coupled device scanner (Leaf Systems, Inc., Westborough, MA) mounted on an Olympus BH-2 microscope. Images were acquired and processed with Adobe Photoshop 4.01 (Adobe Systems, Inc, San Jose, CA) and printed with a Kodak 8650 dye sublimation printer (Kodak Scientific Imaging Systems, Rochester, NY).

Data Analysis

Analyses of LIVE/DEAD and TUNEL images were performed with Optimas 5.22 software (Optimas Corp., Bothell, WA). Green (live) and red (dead) fluorescence images and red (TUNEL-positive) and blue (TUNEL-negative) bright-field images of cells were identified with color sampling, and their number was recorded as total number of corresponding color areas extracted from an image. Values are means $\pm$ SD of five representative areas of a minimum of 200 cells in at least five different microscope fields. Each experiment was performed at least three times.

Electron Microscopy

After removal of the medium, the cells were fixed with 1% paraformaldehyde, 2.0% glutaraldehyde in 0.15 mol/L cacodylate buffer. They were postfixated in 1% osmium tetroxide in 0.15 mol/L cacodylate buffer for 1 hour, washed in distilled water, dehydrated in a graded series of ethanol, stained *en bloc* with 0.5% uranyl acetate in 70% ethanol, and embedded in PolyBed 812/Araldite resin (Polysciences Inc., Warrington, PA).

Cell Death Detection Enzyme-Linked Immunosorbent Assay (ELISA)

The Cell Death Detection ELISA Plus from Boehringer Mannheim was used to measure histone-bound DNA fragments in an ELISA format. Medium was collected at 36 hours after thermal injury and used directly in this assay.

DNA Laddering

DNA was isolated from cells cultured on coverslips or media with DNAzol BD (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instruc-

tions. DNA was biotinylated with the TACS Apototic DNA laddering kit (Trevigen, Gaithersburg, MD), electrophoretically separated on a 1.0% agarose gel, and transferred to 0.2 μ m Nytran (Schleicher & Schuell, Keene, NH). Labeled DNA was detected by chemiluminescence (Trevigen) on Kodak Biomax film (Sigma).

Western Blots

Protein and nucleic acids were isolated from cells with the TRI reagent (Molecular Research Center). Protein was precipitated from the phenol phase according to the manufacturer's instructions and solubilized with 2% sodium dodecyl sulfate and 2% β -mercaptoethanol in Tris buffer.²⁹ The protein concentration in each sample was determined by application of 1 μ l to a nitrocellulose membrane, drying, and staining with amido black (Sigma). The membrane was rinsed, dried, and digitized with a scanner, and the amount of protein was measured by densitometry and referenced to known amounts of bovine serum albumin similarly spotted onto the membrane. Equal amounts of protein from thermally injured cells were separated on 4 to 12% polyacrylamide gels (Novex, San Diego, CA), electrophoretically transferred to polyvinylidene fluoride membrane, and probed with antibody to the corresponding antigen. For immunochemical detection of hsp70 and p53, the membrane was blocked with 2% casein and incubated with monoclonal anti-hsp70 (Stressgen, Vancouver, BC, Canada) or anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by reaction with alkaline phosphatase-conjugated goat anti-mouse antibody (DAKO Corp., Carpinteria, CA). After removal of unbound secondary antibody, the blot was developed for alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Zymed, San Francisco, CA) to localize hsp70 and p53. Relative amounts of hsp70 and p53 were quantified by integrated densitometric determination of the bands using a microcomputer and Gel Pro software (Media Cybernetics, Silver Spring, MD).

Results

LIVE/DEAD and TUNEL assays were performed sequentially on the same NHEKs up to 72 hours after heating to various temperatures for 1 second. Figure 1 presents typical fluorescence (LIVE/DEAD assay) and bright-field (TUNEL) micrographs taken 24 hours after thermal injury. Control cells were all alive and TUNEL negative (Figure 1, A and B). After treatment at 58°C (Figure 1, C and D), all cells remained viable, but some were TUNEL positive (small arrowheads), indicating apoptosis. After exposure to 60°C (Figure 1, E and F), the number of cells degrading their DNA increased and only half the TUNEL-positive cells appeared viable (small arrowheads), whereas the rest were ethidium homodimer-1 positive (large arrowheads). After a 62°C exposure (Figure 1, G and H), most of the cells were TUNEL positive and ethidium homodimer-1 positive. Cells tended to round up and detach from the substrate, and many of them were lost during

Figure 1. Simultaneous LIVE/DEAD and TUNEL assays for discriminating between apoptosis and ACD. (Right and left micrographs are of the same cells.) Photomicrographs are of control NHEKs (A and B) and NHEKs exposed to 58°C (C and D), 60°C (E and F), 62°C (G and H), and 72°C (I and J) for 1 second each, 24 hours posttreatment. Staining was with the LIVE/DEAD Viability/Cytotoxicity Kit (A, C, E, G, and I) to differentiate live (green) from dead (red) cells followed by TUNEL labeling (B, D, F, H, and J) to indicate cells with fragmented DNA (red). **Small arrowheads:** Viable cells with fragmented DNA. **Large arrowheads:** Dead TUNEL-positive cells.

Figure 2. Cell death after thermal injury. NHEKs were exposed to 58°C (A), 60°C (B), 62°C (C), or 72°C (D) for 1 second. Data are expressed as a percentage of TUNEL-positive cells (■) and ethidium homodimer-1-positive cells (●). Data from at least three experiments were pooled. Values are mean $\pm$ SD of five representative areas of a minimum of 200 cells in at least five different microscope fields.

processing of the coverslips. NHEKs heated to 72°C for 1 second (Figure 1, J and K) were all ethidium homodimer-1 positive and TUNEL positive. The outline of the cell was retained indefinitely after heating because of a form of heat fixation, perhaps similar to the coagulation necrosis, observed *in vivo* after severe burn injury.

Although we assume that TUNEL-positive viable cells are undergoing apoptosis, cells with plasma membrane permeable to ethidium homodimer-1 and fragmented DNA could be in the late stages of apoptosis or dying by ACD. If DNA degradation occurs before loss of plasma membrane integrity, we suppose that that cell is dying by apoptosis. If these two processes begin simultaneously or plasma membrane becomes permeant before DNA is fragmented, cells are dying by ACD. Because we cannot detect the order of the events in a dying cell by looking at cell images taken at one time point, we studied the kinetics of the processes in populations of NHEKs.

The results were quantified as a function of time after heating and are presented as a percentage of keratinocytes in the process of cell death registered by two criteria: viability loss (dead cells) and DNA degradation (TUNEL-positive cells) (Figure 2). It appeared that immediately after treatment at 58 and 60°C (Figure 2, A and B), DNA degradation and loss of viability occurred simultaneously in a small portion of cells, and by 6 hours the number of cells undergoing ACD reached about 5%. Afterward, DNA fragmentation became the leading process in populations of dying cells. After exposure to 58°C, the number of nonviable cells did not increase until

Figure 3. Detection of histone-associated DNA fragments with *in situ* cell death ELISA. NHEK cell culture medium was analyzed 36 hours after heating to temperatures of 56 to 62°C for 1 second. Data are shown as optical density at 405 nm for 2,2'-azino-di[3-ethylbenzthiazolin-sulfonate] supplied as substrate for peroxidase conjugated to monoclonal anti-DNA antibody. Values are mean $\pm$ SD of nine samples of medium from wells containing coverslips with NHEKs.

48 hours after injury. After 72 hours, around 30% of cells could be identified as apoptotic, and only 5% had permeable plasma membranes. It appeared that cells die predominantly by apoptosis, and it may take up to 6 days to complete the process.

Figure 4. Detection of DNA laddering by Southern blot of DNA isolated from medium. DNA was isolated from NHEK cell culture medium 36 hours after heating to temperatures of 56°C (A), 58°C (B), or 59°C (C) for 1 second and labeled with biotin dCTP followed by streptavidin alkaline phosphatase and chemiluminescence detection with diiodetane. Arrowheads indicate size of DNA fragments in base pairs.

Figure 5. MitoTracker Orange evaluation of mitochondrial transmembrane potential 24 hours after thermal injury. Photomicrographs are of control NHEKs (A, D, G, and J) and cells heated to 58°C (B, E, H, and K) or 62°C (C, F, I, and L). Staining with calcein AM (A to C) to identify live (green) cells and MitoTracker Orange (D to F) to visualize functional mitochondria (bright orange) was followed by TUNEL labeling (G to I) to mark cells with fragmented DNA (red). J to L: Staining pattern of a single cell with MitoTracker Orange. **Arrowheads** indicate live, TUNEL-positive cells with functional mitochondria (B, E, and H) or after mitochondrial permeability transition (C, F, and I).

After treatment at 60°C, around 70% of cells were TUNEL positive after 72 hours, with more than 40% of all cells being nonviable. If kinetics of the events in apoptosis after 60°C exposure is not different from that after 58°C treatment, it appears that about half the cells could be dying by apoptosis and half by ACD. It is possible that some cells initiate apoptosis but cannot complete it, because they are incapable of the required gene expression.

After exposure to 62°C (Figure 2C), ACD was the predominant form of cell death; most cells were ethidium homodimer-1 positive before they became TUNEL positive. After exposure to 72°C (Figure 2D), however, all cells became ethidium homodimer positive-1 immediately, but they became TUNEL positive only after several hours in the medium. This late acquisition of TUNEL positivity was probably the result of spontaneous degradation of DNA.

Figure 3 illustrates the pattern of release of fragmented DNA into the medium 36 hours after treatment at various temperatures. In this ELISA, the capture antibody binds histone and the reporter system is an anti-DNA antibody conjugated to alkaline phosphatase. Maximum release occurred after treatment to 58 and 59°C.

To determine whether the ELISA actually detected nucleosomal fragments, DNA was extracted from the medium and electrophoresed on agarose gels. Because of the small amounts of DNA, standard staining of gels could not be used. To visualize the electrophoretic DNA patterns, the isolated DNA was prelabeled and transferred to a nylon membrane where chemiluminescence detection was applied. Figure 4 illustrates that nucleosome-sized fragments were released into the medium 36 hours after treatment at 58°C. Very little DNA was detected in the medium from either control cells or cells

Figure 6. Transmission electron micrographs of control NHEKs and thermally injured NHEKs. Shown are control NHEKs (A) and NHEKs 24 hours after 1-second exposure to 58°C (B), 59°C (C), or 60°C (D). Bars indicate 1.0 μm.

Figure 7. Transmission electron micrographs of mitochondria in control or thermally injured NHEKs. Shown are mitochondria in control NHEKs (A), in NHEKs 20 hours after exposure to 58°C for 1 second (B), and 12 hours (C) or 24 hours (D) after 1-second exposure to 60°C. Bars indicate 1.0 μ m.

heated to higher temperatures. This result confirmed that DNA fragmentation detected in the ELISA was actually nucleosome sized. The ELISA assay was negative at 12 and 24 hours, indicating that degradation is a late event in keratinocyte apoptosis.

Figure 5 shows the results of combining the MitoTracker Orange probe of mitochondrial permeability transition with calcein staining and the TUNEL assay. All control cells were calcein positive, MitoTracker Orange positive, and TUNEL negative (Figure 5, A, D, G, and J). Twenty-four hours after a 1-second exposure to 58°C (Figure 5, B, E, H, and K), some TUNEL-positive, but still viable cells exhibited a punctate orange staining pattern (Figure 5, large arrowheads). This staining indicates that keratinocytes began undergoing DNA fragmentation before the mitochondrial permeability transition. After exposure to 62°C (Figure 5, C, F, I, and L), a temperature shown previously to cause predominantly ACD, all TUNEL-positive cells had undergone the mitochondrial permeability transition, although some of these cells are still viable by calcein staining (Figure 5, small arrowheads).

At the ultrastructural level (Figure 6), NHEKs undergoing apoptosis exhibited changes that have also been observed in other cell types: cell shrinkage, cytoplasmic budding, and alterations in nuclear morphology. Twenty-four hours after exposure to 58°C, peripheral aggregations of nuclear chromatin and breakup of the nucleus was evident. Twenty-four hours after exposure to 59°C, the cells underwent chromatin condensation, but alterations in nuclear morphology were not yet apparent. In contrast, cells treated at 60°C undergo ACD instead of apoptosis; at 24 hours after treatment, the cells did not exhibit the nuclear alterations that apoptotic cells did. Nuclear chromatin became condensed, but aggregation of chromatin and fragmentation of nuclei did not occur. The cytokeratin filaments became aggregated in perinu-

clear zones, and the outer rim of cytoplasm was devoid of shape. These cells did not seem to swell as occurs in the oncosis that follows ischemic injury.⁹

Ultrastructural changes in mitochondria after heating are shown in Figure 7. Twenty-four hours after heating to 58°C, the mitochondria were thinner and denser than controls, but still MitoTracker Orange positive. Twelve hours after heating to 60°C, however, mitochondria showed significant shrinkage but intact cristae, and by 24 hours they were MitoTracker Orange negative and the cristae were obliterated.

The process of apoptosis after thermal injury is relatively asynchronous, and many morphological stages were observed simultaneously. Figure 8 presents keratinocytes heated to 58°C for 1 second at 12 and 24 hours to show the typical features of cells in the execution stage of apoptosis. Figure 8A is a LIVE/DEAD analysis that illustrates a live cell in the process of budding and completing the execution phase of apoptosis. Figure 8, B and C, exhibit the ultrastructural features of this process at 12 and 24 hours. These features of apoptosis are not often observed in TUNEL-stained specimens, as they are loosely attached and lost during the processing.

A defining feature of apoptosis is that it is considered to be gene directed. Therefore, thermally injured keratinocytes should retain the capacity for macromolecular synthesis. To test the hypothesis, the synthesis of hsp70 and p53 was examined. Figure 9 shows hsp70 and p53 levels 8 hours after a 1-second treatment with various doses of heat. hsp70 exhibited significantly enhanced synthesis compared with control after treatment at 57 to 58°C, whereas higher temperatures resulted in decreased synthesis. A maximum synthesis of p53 occurred after exposure to 58°C. These results indicate that cells exposed to 58°C are capable of macromolecular synthesis and that apoptosis is one response to this injury.

Figure 8. Morphological changes in thermally injured keratinocytes (58°C for 1 second). **A:** Fluorescence LIVE/DEAD image taken 24 hours posttreatment. One cell undergoing budding and three rounded-up cells can be identified. **B and C:** Transmission electron micrographs of cells undergoing budding at 12 hours (**B**) and 24 hours (**C**) after thermal injury. Bars indicate 1.0 μm .

Discussion

Several studies have shown that skin keratinocytes undergo apoptosis during normal development and also as part of their program of terminal differentiation.^{30,31} This study describes the induction of apoptosis in normal human keratinocytes after a short pulse of relatively high temperature. These results correlate with other studies that indicate that the mode of cell death—whether apoptosis or ACD—is a function of intensity of insult.^{32,33}

Figure 9. Synthesis of hsp70 and p53 in NHEKs, as a function of temperature, 8 hours posttreatment. **A, top:** Western blot of hsp70; graph shows the relative amount of hsp70 detected by densitometry. **B, top:** Western blot of p53; graph shows the relative amount of p53 detected by densitometry.

Very high temperature exposure results in immediate death by a form of fixation.

Following the recommendation of Majno and Joris⁹ and Trump et al,³⁴ we have reserved the term “apoptosis” for that mode of cell death occurring in cells with an intact plasma membrane, mitochondria, and protein synthetic apparatus. A more satisfactory method than those used currently for discriminating between apoptosis and ACD couples a viability assay with a DNA damage test. This method provides evidence that the mode of cell death is apoptosis rather than ACD, as apoptosis requires the active participation of the targeted cell. If the cell is alive and TUNEL positive, it is probably undergoing apoptosis.

A dead, TUNEL-positive cell that does not exhibit nuclear or cytoplasmic fragmentation probably died by ACD.

Morphological features of apoptosis that most cell types exhibit include decreased cell volume and marked shape changes with budding. By electron microscopy, apoptotic cells exhibit relatively intact but shrunken mitochondria, aggregation of chromatin, breakup of the nucleus, and generation of pseudopodia (budding). These characteristics were also observed here after thermal injury in NHEKs after exposure in the range of 57 to 59°C. The degradation of DNA to nucleosome-sized fragments after exposure to 58 and 59°C was a very late event, occurring perhaps at or about the time the cells rounded up and detached from the substrate. This delay in DNA fragmentation may be a peculiarity of keratinocytes, or it may indicate that thermal injury results in a lag in the execution phase of apoptosis. After exposure to temperatures above 60°C, ACD was the predominant mode of cell death; ie, most cells became ethidium homodimer-1 positive and TUNEL positive simultaneously. This pattern did not change much after treatment at 62 to 66°C, although cells detached more rapidly at these higher temperatures, reflecting perhaps denaturation of cellular integrins or extracellular matrix attachment proteins. Cell loss into the medium at these higher temperature exposures was high, and it was therefore difficult to quantify changes in viability and TUNEL staining in these cells.

Loss of mitochondrial transmembrane potential, as determined with MitoTracker Orange, preceded overt nuclear signs of apoptosis, and it may be used instead of the LIVE/DEAD assay in conjunction with TUNEL to discriminate between apoptosis and ACD in a variety of cell types. Mitochondrial permeability transition has also been observed to precede DNA fragmentation and involves opening of the mitochondrial megachannels, allowing free distribution of solutes <1500 d on both sides of the inner mitochondrial membrane.¹⁷⁻²³ This alteration in membranes results in loss of the proton gradient and uncoupling of oxidative phosphorylation. Because this dye can be covalently bound to cells with aldehydes, it could be assessed after fixation and used in conjunction with the TUNEL assay. A viability assay would then not be required, nor would the same area of a specimen need to be evaluated a second time.

Degradation of nuclear DNA into a characteristic pattern of nucleosomal fragments is considered by many to be the hallmark of apoptosis.³⁵ In this study, we used the most sensitive tests available for examining degradation of DNA to nucleosome-sized fragments, because only small amounts of DNA were available. Equivocal results were obtained with *in situ* cell death ELISA when the cell layer was extracted per the protocol instructions, but they were reproducible when the medium was examined directly. Because the ELISA only measures DNA-histone complexes, we confirmed that it was measuring nucleosomes by gel electrophoresis. These results demonstrated that DNA degradation to nucleosome-sized fragments occurred in keratinocytes after thermal injury and was a late event, as it was not detected earlier than 20 hours posttreatment.

Although apoptosis is considered to be gene directed, the nature of the expressed genes is largely unknown. The family of cysteine proteases, which play prominent roles in apoptosis, are preformed proenzymes, and they act on each other in a proteolytic cascade once apoptosis is triggered; they do not have to be synthesized. hsp70 synthesis was elevated after exposure to temperatures up to 58°C for 1 second but not at higher temperatures.

The phosphoprotein p53, best known as a tumor suppressor, normally accumulates after DNA damage, and it has been suggested to play a role in induction of apoptosis in ultraviolet light-irradiated keratinocytes.³⁶ Its synthesis here was elevated above control after exposure to 58°C but not higher temperatures. Thermal injury is not known to induce DNA damage directly, but p53 was synthesized here in thermally injured, yet viable cells. Cells that did not respond in this way, ie, those heated to >59°C, probably cannot undergo apoptosis and die by ACD. Our results are consistent with the role of p53 in inducing apoptosis.

The results reported here strongly suggest that apoptosis occurs in epidermis *in vivo* after burn injury when the basal cell layer is heated to 58 to 59°C and that ACD occurs after injury from higher temperatures. Finding ways to rescue thermally injured, yet viable, keratinocytes would accelerate the wound-healing process by retaining more cells in the pool of repairing cells.

References

1. Pruitt BA, Goodwin CW, Pruitt SK: Burns: including cold, chemical, and electric injuries. Textbook of Surgery. Edited by DCJ Habiston. Philadelphia, WB Saunders Co, 1991, pp 178-209
2. Langer R, Vacanti JP: Tissue Engineering Science 1993, 260:920-926
3. Gerweck LE: Hyperthermia in cancer therapy: the biological basis and unresolved questions. Cancer Res 1985, 45:3408-3414
4. Dewey WC: Failla memorial lecture. The search for critical cellular targets damaged by heat. Radiat Res 1989, 120:191-204
5. Dewey WC: Arrhenius relationships from the molecule and cell to the clinic. Int J Hypertherm 1994, 10:457-483
6. Laszlo A: The effects of hyperthermia on mammalian cell structure and function. Cell Proliferation 1992, 25:59-87
7. Moritz AR, Henriques FCJ: Studies of thermal injury. II. The relative importance of time and surface temperature in the causation of cutaneous burns. Am J Pathol 1947, 23:695-720
8. Bowman PD, Schuschereba ST, Lawlor DF, Gilligan GR, Mata JR, Debaere DR: Survival of human epidermal keratinocytes after short-duration high temperature: synthesis of HSP70 and IL-8. Am J Physiol 1997, 272 (Cell Physiol:41):C1988-C1994
9. Majno G, Joris I: Apoptosis, oncosis, and necrosis: an overview of cell death (see comments). Am J Pathol 1995, 146:3-15
10. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992, 119:493-501
11. Charriaut-Marlangue C, Ben-Ari Y: A cautionary note on the use of the TUNEL stain to determine apoptosis. Neuroreport 1995, 7:61-64
12. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R: *In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 1995, 21:1465-1468
13. Nakamura M, Yagi H, Kayaba S, Ishii T, Ohtsu S, Itoh T: Most thymocytes die in the absence of DNA fragmentation. Arch Histol Cytol 1995, 58:249-256

14. Cervos-Navarro J, Schubert T: Pitfalls in the evaluation of apoptosis using TUNEL. *Brain Pathol* 1996, 6:347-348
15. Van Lookeren Campagne M, Gill R: Ultrastructural morphological changes are not characteristic of apoptotic cell death following focal cerebral ischaemia in the rat. *Neurosci Lett* 1996, 213:111-114
16. de Torres C, Munell F, Ferrer I, Reventos J, Macaya A: Identification of necrotic cell death by the TUNEL assay in the hypoxic-ischemic neonatal rat brain. *Neurosci Lett* 1997, 230:1-4
17. Hirsch T, Marzo I, Kroemer G: Role of the mitochondrial permeability transition pore in apoptosis. *Biosci Rep* 1997, 17:67-76
18. Kroemer G, Zamzami N, Susin SA: Mitochondrial control of apoptosis. *Immunol Today* 1997, 18:44-51
19. Macho A, Decaudin D, Castedo M, Hirsch T, Susin SA, Zamzami N, Kroemer G: Chloromethyl-X-Rosamine is an aldehyde-fixable potential-sensitive fluorochrome for the detection of early apoptosis. *Cytometry* 1996, 25:333-340
20. Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeflner A, Hirsch F, Geuskens M, Kroemer G: Mitochondrial permeability transition is a central coordinating event of apoptosis. *J Exp Med* 1996, 184:1155-1160
21. Marchetti P, Susin SA, Decaudin D, Gamen S, Castedo M, Hirsch T, Zamzami N, Naval J, Senik A, Kroemer G: Apoptosis-associated derangement of mitochondrial function in cells lacking mitochondrial DNA. *Cancer Res* 1996, 56:2033-2038
22. Marchetti P, Decaudin D, Macho A, Zamzami N, Hirsch T, Susin SA, Kroemer G: Redox regulation of apoptosis: impact of thiol oxidation status on mitochondrial function. *Eur J Immunol* 1997, 27:289-296
23. Petit PX, Zamzami N, Vayssiere JL, Mignotte B, Kroemer G, Castedo M: Implication of mitochondria in apoptosis. *Mol Cell Biochem* 1997, 174:185-188
24. Lotem J, Sachs L: Differential suppression by protease inhibitors and cytokines of apoptosis induced by wild-type p53 and cytotoxic agents. *Proc Natl Acad Sci USA* 1996, 93:12507-12512
25. Magal SS, Jackman A, Pei XF, Schlegel R, Sherman L: Induction of apoptosis in human keratinocytes containing mutated p53 alleles and its inhibition by both the E6 and E7 oncoproteins. *Int J Cancer* 1998, 75:96-104
26. Barlow Y, Pye RJ: Keratinocyte culture. *Animal Cell Culture*, vol 5, *Methods in Molecular Biology*. Edited by JW Pollard, JM Walker. Clifton, NJ, The Humana Press 1990, pp 83-97
27. Tsao MC, Walthall BJ, Ham RG: Clonal growth of normal human keratinocytes in a defined medium. *J Cell Physiol* 1982, 110:219-229
28. Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillemet C, Brambilla C, Brambilla E: In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. *J Histochem Cytochem* 1996, 44:959-968
29. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227:680-685
30. McCall CA, Cohen JJ: Programmed cell death in terminally differentiating keratinocytes: role of endogenous endonuclease. *J Invest Dermatol* 1991, 97:111-114
31. Polakowska RR, Piacentini M, Bartlett R, Goldsmith LA, Haake AR: Apoptosis in human skin development: morphogenesis, periderm, and stem cells. *Dev Dyn* 1994, 199:176-188
32. Dypbukt JM, Ankarcrona M, Burkitt M, Sjöholm A, Strom K, Orrenius S, Nicotera P: Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells: the role of intracellular polyamines. *J Biol Chem* 1994, 269:30553-30560
33. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA: Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci USA* 1995, 92:7162-7166
34. Trump BF, Berezesky IK, Chang SH, Phelps PC: The pathways of cell death: oncosis, apoptosis, and necrosis. *Toxicol Pathol* 1997, 25: 82-88
35. Bortner CD, Oldenburg NBE, Cidlowski JA: The role of DNA fragmentation in apoptosis. *Trends Cell Biol* 1995, 5:21-26
36. Henseleit U, Zhang J, Wanner R, Haase I, Kolde G, Rosenbach T: Role of p53 in UVB-induced apoptosis in human HaCaT keratinocytes. *J Invest Dermatol* 1997, 109:722-727