Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes

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SUMMARY

We show that mouse sperm die spontaneously within 1-2 days in culture and that treatment with either staurosporine (STS) and cycloheximide (CHX) or a peptide caspase inhibitor does not accelerate or delay the cell death. Chicken erythrocytes, by contrast, are induced to die by either serum deprivation or treatment with STS and CHX, and embryonic erythrocytes are more sensitive than adult erythrocytes to both treatments. Although these erythrocyte deaths display a number of features that are characteristic of apoptosis, they are not blocked, or even delayed, by peptide caspase inhibitors, and most of the cells die without apparently activating caspases. A small proportion of the dying erythrocytes do activate caspase-3, but even these cells, which seem to be the least mature erythrocytes, die just as quickly in the presence of caspase inhibitors. Our findings raise the possibility that both mouse sperm and chicken erythrocytes have a death programme that may not depend on caspases and that chicken erythrocytes lose caspases as they mature. Chicken erythrocytes may provide a useful ‘stripped down’ cell system to try to identify the protein components of such a death programme, which may serve to back-up the conventional caspase-dependent suicide mechanism in many cell types.

Key words: Cell death, Chicken, Erythrocyte, Sperm, Caspase
raising the possibility that they reflect the operation of a caspase-independent death programme.

MATERIALS AND METHODS

Animals, cells, and reagents

Sprague-Dawley rats and Balb/c × C57Bl/6 F1 mice were purchased from either Harlan UK Ltd (Bicester, UK) or the University College London Animal Facility. White Leghorn chicken eggs were purchased from JK Needle and Co. (Herts, UK), while fresh adult chicken blood was purchased from Harlan Sera-Lab (Loughborough, UK). The DT-40 chicken lymphoma cell line was provided by S. Moss (University College London). Rat thymocytes were prepared from postnatal day 14 (P14) Sprague-Dawley rats. All reagents were from Sigma unless otherwise stated. The peptide caspase inhibitors BocD-fmk (Boc-Asp(O-Me)-CH2F) and zVAD-fmk (Cbz-Val-Ala-Asp (O-Me)-CH2F), and the fluorogenic peptide substrates zDEVD-afc (Cbz-Asp-Glu-Val-Ala-Asp-amidotri fluoromethylcoumarin), zVEID-afc (Cbz-Val-Glu-Ile-Asp-afc), and zVAD-afc (Cbz-Val-Ala-Asp-afc) were purchased from Enzyme Systems Products, Inc. (Livermore, CA, USA). The caspase inhibitor zD-CH2-DCB (Cbz-Asp-CH2-[2,6-dichlorobenzoyloxy]methylene) was purchased from Calbiochem-Novabiochem (Nottingham, UK). Stock solutions of peptide inhibitors and substrates were made up in dry DMSO (the inhibitors at 50 mM and the substrates at 20 mM) and stored in aliquots at −80°C.

Fluorescent annexin-V was prepared by labelling purified recombinant annexin-V with fluorescein isothiocyanate (FITC) using standard techniques (Harlow, 1988). Purified recombinant annexin-V was prepared by bacterial expression of a GST-annexin-V fusion protein (using pGEX-annexin-V transformed bacteria provided by S. Moss, University College London), followed by purification on glutathione-agarose beads (Smith and Johnson, 1989).

Mouse sperm

Mouse sperm were collected from adult male mice. Epididymides were removed, punctured with a needle, and squeezed gently to expel the sperm into 6 ml of Biggers-Whitten-Whittington medium (BWWM) (Biggers et al., 1971), containing 30 mg/ml bovine serum albumin (BSA). The sperm were washed twice in BWWM without BSA, counted in a haemocytometer, and 5×10^5 cells in 500 μl of BWWM were added to each well of a 24-well culture dish (Falcon), in the presence or absence of STS and CHX, at a final concentration of 10 μM and 10 μg/ml, respectively. In some wells, the peptide caspase inhibitor zVAD-fmk was added to a final concentration of 100 μM. The cells were cultured for 24 hours at 37°C in 5% CO2 and cell survival was assessed by simultaneous staining for 10-20 minutes at 37°C with bisbenzimide to label all cells, calcein-AM to label the cytoplasm of live cells (i.e., cells with an intact plasma membrane), and propidium iodide to label dead cells. A 20 μl drop of the triple-labelled sample was analysed by fluorescence microscopy, as described above. In some experiments the presence of phosphatidylserine on the cell surface, a characteristic feature of apoptosis (Fadok et al., 1992), was assessed by staining for 40 minutes at 37°C with annexin-V-FITC (Martin et al., 1995); 0.1 μg/ml, together with bisbenzimide and propidium iodide.

TUNEL staining

Cultured or fresh chicken erythrocytes were double labelled with bisbenzimide and propidium iodide as described above. The cells were then washed twice in 5% normal goat serum (NGS) in PBS and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The cells were then labelled by the TdT-mediated dUTP-biotin nick end-labelling (TUNEL) technique (Gavrieli et al., 1992), using a fluorescence detection kit (Boehringer, Mannheim).

Immunodetection of activated caspase-3

To detect the activated form of caspase-3 we used affinity-purified rabbit antibodies (CM1, kindly supplied by Anu Srinivasan, Idun Pharmaceuticals, Inc.) raised against a 13-amino-acid peptide corresponding to the C terminus of the large (p20) subunit of activated human and mouse caspase-3 (Armstrong et al., 1997). In western blots, these antibodies recognise the activated form of caspase-3, but not the inactive pro-enzyme (Armstrong et al., 1997; Anu Srinivasan, Idun Pharmaceuticals, Inc., personal communication).

Paraformaldehyde-fixed cells, which had been prelabelled with bisbenzimide and propidium iodide as described above, were washed twice in PBS, incubated for 1 hour in blocking buffer (10% NGS, 0.4% Triton X-100 in PBS) at room temperature and then for 1 hour in 260 ng/ml of the CM1 antibodies in incubation buffer (2% NGS, 0.4% Triton X-100 in PBS). The cells were washed 3 times with 0.1% Tween-20 in PBS, and the bound antibodies were detected with biotinylated anti-rabbit immunoglobulin antibodies (Amersham; diluted 1:200 in incubation buffer), followed by streptavidin-FITC (Amersham; diluted 1:200). The cells were then resuspended in PBS and examined by fluorescence microscopy.

DNA electrophoresis

DNA was extracted from 20×10^6 sperm or erythrocytes as described by Martin and Green (1996), and 3 μg of DNA was electrophoresed in a 1.8% TBE/agarose gel, which was then stained with ethidium bromide and visualised under UV light. Cells dissociated from a mouse thymus were used as a positive control; the cells were treated with STS (1 μM) and CHX (10 μg/ml) in DMEM for 16 hours at 37°C, and the DNA was extracted and analysed as just described.

Cytosolic extracts

Cytosolic extracts were prepared from chicken erythrocytes, rat thymocytes, or DT40 cells. About 15×10^6 erythrocytes, 5×10^6 thymocytes, or 2.5×10^8 DT40 cells were cultured for various times in either a 10 cm culture dish (Sterilin, UK) or a tissue culture flask (Falcon, UK), with or without STS (5 μM) and CHX (10 μg/ml). EDTA (0.5 mM) was added to the cultures just before the cells were collected. The cells were washed by centrifugation in PBS-0.5 mM EDTA, and an aliquot was taken to assess cell viability by staining with bisbenzimide and propidium iodide as described above. 0.1% detergent extracts were prepared by sonication on ice, as described by Liu et al. (1996), with some modifications: the cells were resuspended (without sonication) in ice-cold buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM Na EDTA, 1 mM Na EGTA, 1 mM dithiothreitol), supplemented with protease inhibitors (0.1 mM
phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 25 μg/ml n-acetyl-leu-leu-norleucinal). After sonication, the extracts were centrifuged at 14,000 rpm in a Microfuge (Eppendorf 5415) for 5 minutes at 4°C. The supernatants were further centrifuged at 45,000 rpm for 30 minutes at 4°C in a Beckman tabletop ultracentrifuge, using a TLA-45 rotor. The protein concentrations of the resulting supernatants were assayed using the Coomassie Plus Protein Assay Reagent (Pierce, USA), and the supernatants were stored in aliquots at ~80°C until use.

Caspase activity
To detect the activity of caspases, 110 μg of erythrocyte extract or 5-10 μg of thymocyte or DT40 extract was incubated in buffer A (see above) and 2 mM additional MgCl₂, with 0.1 mM fluorogenic peptide caspase substrate (either zDEVD-AFC, VEID-AFC, or zVAD-AFC) at 37°C. In some cases, dATP and cytochrome c were added to activate specific procaspases in the extract (Liu et al., 1996). Aliquots (25 μl) were taken at 0, 15, 30, 45, 60, 120 and 240 minutes and were added to an Eppendorf tube containing 12.5 μl of a solution of 1% Na acetate. 3H₂O in 175 mM acetic acid to stop the reaction. The samples were stored on ice until the end of the experiment, at which time 462.5 μl of 0.1% Chaps in water was added to all samples, which were then centrifuged at 5,000 g for 1 hour at 40°C. All samples were filtered through Centricon 10 filters (Amicon, Inc., Beverly, USA) to remove the haemoglobin (which otherwise quenched the fluorescence signal) from samples containing erythrocyte extract. The samples were then stored at −200°C until analysis. The amount of cleaved AFC in each of three 150 μl aliquots from each sample was measured in a 96-well plate luminescence spectrometer (Perkin Elmer LS 50B; excitation at 400 nm and emission at 505 nm). Varying concentrations of free AFC were used to plot a standard fluorescence curve, subtracting the fluorescence value of a water blank from each AFC value. The fluorescence value of a sample taken at the same time point from a control reaction mixture that contained no cell extract was subtracted from the fluorescence value of the experimental sample. The specific activity of caspases in an extract was defined as the maximum rate of AFC released (Kmax in pmol/hour), divided by the amount of protein extract (in μg) in each well, where pmol AFC release for each time point was converted from fluorescence values based on the standard AFC curve done in every experiment, and the Kmax was determined from the maximum slope of the plot of pmol AFC release vs time.

RESULTS
Death of mouse sperm
Mouse sperm prepared from adult epididymi were cultured for 6, 24 and 48 hours at 37°C in BWWM, which was previously found to be optimal for sperm survival (Biggers et al., 1971). We assessed viability by double-labelling with bisbenzimide to label all cells and propidium iodide to label dead cells. The results are shown as mean ± s.d. of triplicates from a representative experiment. The experiment was repeated three times with similar results. Note that none of the treatments affected cell viability.
shown); only occasional cells were viable after 2 days (not shown). Treatment with either 10 μM STS and 10 μg/ml CHX or 100 μM of the caspase inhibitor zVAD-fmk did not significantly influence sperm viability (Fig. 1). As shown in Fig. 2, the morphology of the nucleus did not change appreciably when a sperm died. None of the dead sperm could be labelled by the TUNEL technique, which identifies cells with fragmented DNA (Gavrieli et al., 1992) (not shown), but even DNase-treated sperm could not be labelled, presumably because of the unusually dense packing of sperm chromatin. We were also unable to detect a ladder pattern of DNA fragments (see below) when DNA was extracted from either fresh sperm or sperm that had been treated for 1 day with STS and CHX and then analysed by gel electrophoresis (not shown).

To determine whether caspase-3 became activated in the dying sperm, we used affinity-purified rabbit antibodies that specifically recognise the activated form of this caspase in indirect immunofluorescence assays. In a freshly isolated population of sperm, where 25% of the cells were dead (propidium iodide positive), only 8% of the dead cells were stained by the anti-caspase-3 antibodies (Fig. 3). Although the proportion of dead sperm increased greatly after 24 hours in culture, with or without STS and CHX treatment, the proportion of total sperm that were stained by the antibody did not increase during this time, suggesting that the majority of sperm cell deaths in culture did not involve the activation of caspase-3.

Death of chicken erythrocytes

Adult or embryonic chicken erythrocytes were washed and cultured in DMEM alone, DMEM plus 10% FCS (DMEM-FCS), or in either of these conditions in the presence of STS and CHX. Aliquots of the cells were assessed for viability after various times by staining with propidium iodide to label dead cells and calcein-AM to label live cells. As shown in Fig. 4A, in DMEM-FCS the great majority of adult erythrocytes survived for up to 10 days, the last time-point examined, and treatment with STS and CHX had little effect; cell survival was reduced, however, in DMEM without FCS, and STS and CHX treatment in these conditions decreased survival further. When embryonic (E10 or E15) chicken erythrocytes were tested in

![Fig. 3](image)

**Fig. 3.** Confocal fluorescence micrograph showing a sperm stained with affinity-purified antibodies that react specifically with activated caspase-3. Freshly isolated sperm were stained with propidium iodide (red) to identify dead cells and then fixed in 4% paraformaldehyde and stained with the antibodies (green), as described in Materials and Methods. Note that only one of the many dead cells is stained by the antibodies. Bar, 10 μm.
the same way, they were found to be more susceptible to the death-promoting effects of both serum-deprivation and STS and CHX treatment. Whereas 70% of E15 erythrocytes survived in DMEM-FCS for 5 days, for example, the last time point examined, less than 40% survived for 5 days in the absence of FCS (Fig. 4B). Moreover, when E15 cells were treated with STS and CHX in DMEM without FCS, all of them died by 3 days. This death was not blocked by the three peptide caspase inhibitors that we tested (BocD-fmk, zV AD-fmk, or zD-CH2-DCB), used either individually (Fig. 4C) or in combination (not shown).

Fig. 5 shows micrographs of E15 chicken erythrocytes analysed by differential interference contrast microscopy (DIC) or by fluorescence microscopy after staining with bisbenzimide, calcein-AM, propidium iodide, or annexin-V-FITC, or by the TUNEL technique. Most of the dead cells showed features that are characteristic of apoptosis: cell-surface staining with annexin-V-FITC (Fig. 5G) occurred early, while loss of plasma membrane integrity, as indicated by propidium iodide staining (Fig. 5D and L) and loss of calcein-AM staining (Fig. 5C), occurred later. The cell and nucleus usually shrunk (Fig. 5A,I,M), and the nucleus was stained by the TUNEL technique (Fig. 5O), suggesting that the DNA was fragmented.

As shown in Fig. 6, when DNA was extracted from E15 cells that had been cultured in DMEM for 36 hours with STS and CHX and the DNA was analysed by gel electrophoresis, a ladder pattern of DNA fragments was seen that is typical of apoptosis (Wyllie et al., 1980). The ladder pattern was not seen if the cells were treated in the same way but in the presence of the caspase inhibitors, used either singly (not shown) or in combination (Fig. 6). Although DNA laddering was blocked by the caspase inhibitors, cell death was not (see Fig. 4C). Moreover, the caspase inhibitors did not inhibit staining with annexin-V-FITC or by the TUNEL technique (not shown).

DNA laddering was also seen when E15 erythrocytes died in response to serum deprivation, and this laddering was blocked by the caspase inhibitors (not shown).

To resolve the paradox that caspase inhibitors blocked DNA laddering but not cell death, we tested directly for caspase activation in two ways, biochemically and immunohistochemically.

**Lack of detectable caspase activity in extracts of dying chicken erythrocytes**

To determine whether caspases activity could be detected when...
E15 or adult chicken erythrocytes died in response to STS and CHX treatment in DMEM without FCS, we made cytosolic extracts of the cells and tested them for their ability to cleave fluorogenic peptide caspase substrates. As can be seen in Table 1, when zDEVD-AFC was used as a substrate, we could not detect caspase activity in extracts of either embryonic or adult erythrocytes that had been treated with STS and CHX long enough to kill more than 50% of the cells, although we could readily detect caspase activity in similar extracts of rat thymocytes treated with STS and CHX for 6 hours, where only 20% of the cells were apoptotic. Similar results were obtained when zVAD-AFC or VEID-AFC were used as substrates (not shown). These findings suggest that caspases that cleave these substrates are not activated when chicken erythrocytes are induced to die by STS and CHX treatment.

To determine if chicken erythrocytes have procaspases that can be activated by cytochrome c and dATP (Li et al., 1997; Liu et al., 1996), we added these reagents to cytosolic extracts of freshly collected E15 erythrocytes. As can be seen in Table 1, this treatment failed to activate caspases that could cleave zDEVD-AFC, and the same result was obtained with extracts of human erythrocytes. The same treatment, however, increased caspase activity in extracts of DT 40 chicken lymphoma cells (Table 1). Similar results were obtained with the two other fluorogenic caspase substrates (not shown). These findings left unresolved the question of why caspase inhibitors blocked the DNA laddering associated with the death of chicken erythrocytes.

**Activated caspases in a minority of dead chicken erythrocytes**

To test the possibility that caspases become activated in a small minority of erythrocytes treated with STS and CHX and that these cells were responsible for the DNA laddering seen, we used affinity-purified rabbit antibodies that react specifically with the large subunit of the activated form of caspase-3, but not with the uncleaved pro-enzyme form of the caspase (Armstrong et al., 1997; Anu Srinivasan, Idun Pharmaceuticals, Inc., personal communication), to assess caspase-3 activation in individual cells by immunofluorescence. When chicken erythrocytes were induced to die by either serum-deprivation or treatment with STS and CHX, only a minority of the dead cells were labelled by the antibodies (Fig. 7). When E15 erythrocytes, for example, were treated with STS and CHX for 2 days so that 90% of the cells were dead (as assessed by propidium iodide staining), about 15% of the dead cells were labelled by the antibodies (Fig. 8). This staining was completely inhibited if the cells were cultured with zVAD-fmk (not shown), confirming that the antibodies only recognize the active form of the caspase.

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**Table 1. Caspase activity in cytosolic extracts of chicken erythrocytes**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment of cells</th>
<th>% Viable*</th>
<th>Treatment of extracts</th>
<th>Caspase activity† pmol AFC/hour per µg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15 chicken erythrocytes</td>
<td>Fresh</td>
<td>100</td>
<td>None</td>
<td>0.92±0.34</td>
</tr>
<tr>
<td>E15 chicken erythrocytes</td>
<td>STS+CHX × 1.5 days§</td>
<td>40</td>
<td>None</td>
<td>1.14±0.48</td>
</tr>
<tr>
<td>Adult chicken erythrocytes</td>
<td>DMEM × 4 days</td>
<td>94</td>
<td>None</td>
<td>0.92±0.46</td>
</tr>
<tr>
<td>Adult chicken erythrocytes</td>
<td>STS+CHX × 4 days§</td>
<td>48</td>
<td>None</td>
<td>0.68±0.32</td>
</tr>
<tr>
<td>Rat thymocytes</td>
<td>Fresh</td>
<td>95</td>
<td>None</td>
<td>4.58±1.08</td>
</tr>
<tr>
<td>Rat thymocytes</td>
<td>STS+CHX × 6 hours§</td>
<td>79</td>
<td>None</td>
<td>224.52±26.37</td>
</tr>
<tr>
<td>E15 chicken erythrocytes</td>
<td>Fresh</td>
<td>100</td>
<td>None</td>
<td>0.92±0.34</td>
</tr>
<tr>
<td>E15 chicken erythrocytes</td>
<td>Fresh</td>
<td>100</td>
<td>Cytochrome c + dATP¶</td>
<td>1.14±0.34</td>
</tr>
<tr>
<td>Human erythrocytes</td>
<td>Fresh</td>
<td>100</td>
<td>Cytochrome c + dATP¶</td>
<td>0.23±0.18</td>
</tr>
<tr>
<td>Human erythrocytes</td>
<td>Fresh</td>
<td>100</td>
<td>Cytochrome c + dATP¶</td>
<td>0.23±0.11</td>
</tr>
<tr>
<td>Chicken DT40 cells</td>
<td>None</td>
<td>72</td>
<td>None</td>
<td>171.82±29.28</td>
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<tr>
<td>Chicken DT40 cells</td>
<td>None</td>
<td>72</td>
<td>Cytochrome c + dATP¶</td>
<td>405.51±65.48</td>
</tr>
</tbody>
</table>

*Cell viability was assessed by double labelling with bisbenzimide and propidium iodide.
†Caspase activity was determined as described in Materials and Methods and is expressed as mean ± s.d. of three independent experiments, except for human erythrocytes and chicken DT40 cells where the results are means ± s.d. of one representative experiment.
§STS was used at 5 µM and CHX at 10 µg/ml.
¶Cytochrome c was used at 10 µg/ml and dATP at 1 mM.
One possibility is that the antibody-stained cells were the most immature erythrocytes, which still had a functional caspase-dependent death programme in place. If this were the case, the proportion of antibody-stained cells would be expected to be greater in populations of dead erythrocytes from younger chickens. As shown in Fig. 8, this was the case: whereas fewer than 10% of dead cells were labelled by the antibodies when adult erythrocytes were induced to die by STS and CHX treatment, this proportion was about 15% in E15 cells and about 35% in E7 cells treated in the same way. Nonetheless, when E7 erythrocytes were treated with STS and CHX for 2 days, more than 95% of the cells died, whether or not caspase inhibitors were present (not shown), suggesting that caspases may not be required for the death, even in the cells where caspase-3 is activated.

**DISCUSSION**

Only recently has it been widely recognised that PCD is a basic property of animal cells. When treated with a high concentration of STS and CHX, for example, all nucleated mammalian cells that have been tested seem to undergo PCD (Weil et al., 1996). Largely on the basis of these findings, we previously proposed that all nucleated animal cells are capable of undergoing PCD and constitutively express all of the proteins required to execute the death programme (Weil et al., 1996). In cases where new RNA and protein synthesis are required for PCD, it was suggested that these are necessary to activate or derepress the death programme rather than to execute it. There is increasing evidence that an intracellular proteolytic cascade involving caspases forms the core of the death programme and that these caspases are constitutively expressed in cells as proenzymes (Chinnaiyan et al., 1997; Cohen, 1997; Nicholson and Thornberry, 1997; Yuan et al., 1993).

Human erythrocytes, which lack a nucleus and other organelles, are the only normal animal cells reported so far that do not seem to have such a death programme: they do not die when either treated with STS and CHX or deprived of survival signals (Weil et al., 1996). In the present study we have analyzed two cell types that contain a nucleus that is transcriptionally inactive and, therefore, might also be expected not to have a death programme, namely sperm and nucleated erythrocytes.

**Sperm death in culture is not blocked by a caspase inhibitor**

Sperm do not survive for long in culture. We find that only 40% of mouse sperm survive for a day in vitro, and only occasional ones survive for 2 days. Treatment with STS and CHX does not accelerate this death, and the peptide caspase inhibitor zVAD-fmk does not retard it. Moreover, the nucleus does not condense when the cells die, perhaps because it is already maximally condensed in the healthy cell. Only a small proportion of the dead sperm are stained by antibodies that specifically recognise the activated form of caspases-3, suggesting that caspase-3, at least, does not become activated when most sperm die in culture.
Although it remains uncertain whether sperm can undergo PCD, it is known that a large proportion of spermatogonia normally die by PCD in the testis (Billig et al., 1995; De Rooij and Lok, 1987). It is possible that sperm, like human erythrocytes, lose their caspase-dependent death programme in their final stages of development and that the small numbers of dead sperm in our experiments that express activated caspase-3 are the least mature of the dead sperm.

**Chicken erythrocyte death in culture may not depend on caspases**

Unlike human erythrocytes, chicken erythrocytes die with a number of features of apoptosis when they are either deprived of serum or treated with STS and CHX. Phosphatidylserine becomes exposed on the cell surface, the cell shrinks, DNA fragments, and plasma membrane integrity is lost relatively late. With maturation, the cells become less sensitive to the death-promoting effects of both serum deprivation and treatment with STS and CHX, but the molecular basis for this maturation change remains to be determined.

Although these erythrocyte deaths resemble apoptosis, several lines of evidence suggest that they may not depend on caspases, at least not on those that are activated in most apoptotic deaths. First, peptide caspase inhibitors do not block or even retard the deaths, and they do not prevent the appearance of phosphatidylserine on the cell surface or the cleavage of DNA revealed by the TUNEL technique (although they do prevent DNA laddering, as discussed below). Second, using several fluorogenic caspase substrates, we cannot detect caspase activity in cytosolic extracts prepared from populations of cells in which more than half of the cells have been induced to die by STS and CHX treatment. Moreover, we cannot detect caspase activity in extracts prepared from freshly isolated erythrocytes after the extracts are treated with cytochrome c and dATP, a treatment that has been shown to activate caspase-9, which, in turn, activates caspase-3, in cytosolic extracts of HeLa cells (Li et al., 1997). This finding suggests that most chick erythrocytes do not express either these caspases, the Ced-4 homologue(s) required for cytochrome c and dATP to activate caspase-9 (Zou et al., 1997), or both. Not surprisingly, we find that cytochrome c and dATP treatment also fails to induce caspase activity in extracts of human erythrocytes. Third, antibodies that recognise the large subunit of activated caspase-3 do not label the great majority of the erythrocytes that die in response to STS and CHX treatment or serum deprivation, suggesting that caspase-3 does not become activated during the death process. Although our experiments suggest that the death of chicken erythrocytes in these circumstances may be caspase-independent, we cannot exclude the possibility that the deaths depend on caspases that are not detected by our assays and are not inhibited by the peptide inhibitors that we used.

**Caspases may become activated in the most immature chicken erythrocytes**

The antibodies that recognise the activated form of caspase-3 do, however, stain a small proportion of the dead chicken erythrocytes. It seems likely that these are the most immature of the dead erythrocytes, as the proportion of stained cells is lower among dead E15 cells than among dead E7 cells, and it is lower still among dead adult cells. It also seems likely that such immature dead erythrocytes are the source of the oligonucleosomal-sized fragments of DNA seen when DNA is extracted from serum-deprived or STS and CHX treated erythrocytes and analysed by electrophoresis, as this DNA laddering is completely blocked by caspase inhibitors, even though the cell death is unaffected. It seems therefore that these immature erythrocytes activate some caspases in the process of dying and that this activation leads to the production of oligonucleosomal-sized DNA fragments but is not required for the cell death. It has recently been shown that caspase-3 triggers DNA fragmentation during PCD, in some cells at least, by cleaving an inhibitory protein that normally holds the responsible DNase in an inactive state (Enari et al., 1998; Sakahira et al., 1998). The simplest interpretation of our findings is that a caspase-dependent death programme is present in immature erythrocytes and is progressively lost as the proteins that mediate the programme, including caspases, are irreversibly lost (as in the absence of protein synthesis proteins cannot be replaced).

**Are there caspase-independent death programmes?**

Does the death of chicken erythrocytes in our experiments reflect the operation of an intrinsic caspase-independent death programme, or does it simply reflect lethal cell dysfunction resulting from harsh treatment? Several findings raise the possibility that the cell deaths result from the activation of a death programme. First, the deaths are associated with a sequence of changes that are more similar to classical PCD than to cell necrosis: phosphatidylserine is displayed on the cell surface early in the process, for example, while plasma membrane integrity is lost late. Second, the deaths are induced by both serum-deprivation and STS and CHX treatment, both of which can induce caspase-dependent PCD in other cell types. Third, the erythrocytes become less sensitive to the death-inducing effects of serum deprivation and STS and CHX treatment with maturation, raising the possibility that these deaths depend on proteins that are progressively lost as the cell ages, although, presumably, they are lost at a slower rate than the proteins that mediate caspase-dependent death in these cells.

There are an increasing number of examples of cell deaths that resemble apoptosis to a greater or lesser extent but seem not to depend on caspases. In one report where cytotoxic T lymphocytes killed target cells, treatment with caspase inhibitors blocked the nuclear changes of apoptosis but did not retard target cell death (Sarin et al., 1997). In cases where the over-expression of Bax in haematopoietic cells (Xiang et al., 1996) or the withdrawal of K+ from cultures of cerebellar granule neurons (Miller et al., 1997) induced apoptosis, caspase inhibitors blocked most of the nuclear changes of apoptosis but not the cell death. In another study, caspase inhibitors delayed but did not stop the death of fibroblasts induced by Bak, Myc, E1A, or etoposide (McCarthy et al., 1997). In one remarkable study the expression of the E4 adenovirus protein E4orfA in rodent fibroblasts induced classical apoptosis without activating caspase-3, and the caspase inhibitor zVAD-fmk failed to inhibit the cell death or any of the features of apoptosis (Lavoie et al., 1998). All of these findings and ours raise the possibility that vertebrate cells have a caspase-independent death programme(s), which may serve as a back-up suicide mechanism. If this is the case,
chicken erythrocytes could provide an attractive ‘stripped down’ cell system in which to identify the components of such a programme.

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