Interdigital cell death can occur through a necrotic and caspase-independent pathway

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Programmed cell death in animals is usually associated with apoptotic morphology and requires caspase activation. Necrosis and caspase-independent cell death have been reported, but mostly in experimental conditions that lead some to question their existence *in vivo*. Loss of interdigital cells in the mouse embryo, a paradigm of cell death during development [1], is known to include an apoptotic [2] and caspase-dependent [3,4] mechanism. Here, we report that, when caspase activity was inhibited using drugs or when apoptosis was prevented genetically (using Hammertoe mutant mice, or mice homozygous for a mutation in the gene encoding APAF-1, a caspase-activating adaptor protein), interdigital cell death still occurred. This cell death was negative for the terminal-deoxynucleotidyl-mediated dUTP nick end-labelling (TUNEL) assay and there was no overall cell condensation. At the electron microscopy level, peculiar 'mottled' chromatin alterations and marked mitochondrial and membrane lesions, suggestive of classical necrotic cell death, were observed with no detectable phagocytosis and no local inflammatory response. Thus, in this developmental context, although caspase activity confers cell death with an apoptotic morphotype, in the absence of caspase activity an underlying mechanism independent of known caspases can also confer cell death, but with a necrotic morphotype. This cell death can go undetected when using apoptosis-specific methodology, and cannot be blocked by agents that act on caspases.

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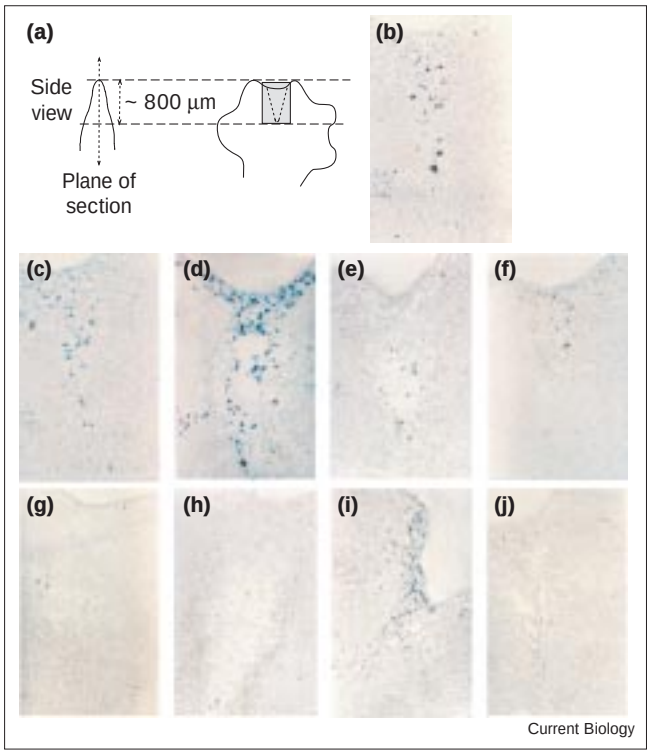
Results and discussion

Interdigital cell death requires bone morphogenetic protein (BMP) receptors [5], members of the transforming growth factor β (TGF-β) receptor family. Their intracellular signal transduction can be increased by treatment with 15-O-desmethyl-FK520 [6]. We incubated mouse limb buds in medium alone or in the presence of 15-O-desmethyl-FK520 for six hours *in vitro*. The presence of cells that were positive for the TUNEL assay (TUNEL$^+$ cells; reflecting DNA fragmentation) or conventionally stained condensed cells, two signs of apoptotic cell death, were checked in both axial (Figure 1a) and transverse (Figure 2a) limb-bud sections; the latter were also viewed by electron microscopy (EM). In the interdigital spaces of limb buds incubated in medium alone, the proportions of TUNEL$^+$ and condensed cells were similar to those seen in freshly fixed samples (Figures 1b,c, 2b and data not shown). When medium-incubated and freshly-fixed samples were viewed under EM, the majority of cells looked normal (Figure 2c) and 5% (2–9%) had an apoptotic morphotype. Incubation with 15-O-desmethyl-FK520 led to more cell-free gaps surrounded by a higher proportion of TUNEL$^+$ (Figure 1d) and condensed cells (data not shown); under EM, 43% (35–51%) of cells had the typical apoptotic morphology (Figure 2d) .

When ZVAD-fmk — a wide-spectrum caspase inhibitor shown to block each of 10 caspases tested [7] — was added together with 15-O-desmethyl-FK520, fewer TUNEL$^+$ and condensed cells were seen, but gaps persisted (compare Figure 1d with Figure 1e); this effect of ZVAD-fmk was seen at 100 μM (and to a lesser extent at 30 μM, data not shown) . Unexpectedly, EM revealed that many of the remaining cells had a morphotype identical to that seen in necrosis [8] including a 'mottled' nucleus caused by clumped, but not marginalised, and only loosely packed chromatin (Figure 2e). By optical microscopy, these necrotic cells could sometimes be seen but were difficult to identify unambiguously and count. Judging by the distinctive appearance of the chromatin in the apoptotic and necrotic morphotypes, as seen under EM, when both 15-O-desmethyl-FK520 and ZVAD-fmk were added, there were 0% (0–5%) apoptotic cells and 30% (23–38%) necrotic cells. In contrast, with 15-O-desmethyl-FK520 only, as mentioned above, 43% apoptotic cells, but only 2% (1–7%) necrotic cells could be found. Incubation with ZVAD-fmk only led to few TUNEL$^+$ (Figure 1f) and condensed cells; by EM, there were 0% (0–5%) apoptotic cells and 6% (3–10%) necrotic cells. Necrotic cells were

Figure 1



Axial sections of mouse interdigital spaces subjected to the TUNEL assay to detect the change in frequency of apoptotic cells resulting from pharmacologic or genetic modulation. TUNEL$^+$ cells appear dark blue. (a) Schematic representation of the plane of axial section. Only sections of the second or third interdigital space are shown. We obtained about 30 serial axial sections per limb bud for a given space. These were heterogeneous as to the presence of TUNEL$^+$ cells; therefore, for each experimental situation and for a given space, the section with the highest proportion of TUNEL$^+$ cells is shown. (b–f) Limb buds from NMRI mice were (b) fixed fresh, or (c) incubated for 6 h *in vitro* in control medium with 0.1% dimethylsulfoxide (DMSO) as a solvent control (the results were similar in medium without DMSO), or in the presence of (d) 15-O-desmethyl-FK520, (e) 15-O-desmethyl-FK520 and ZVAD-fmk or (f) ZVAD-fmk. (g,h) Hind limb buds from the Hammertoe mouse mutant incubated for 6 h *in vitro* in (g) control medium or (h) in the presence of 15-O-desmethyl-FK520. (i,j) Freshly fixed limb buds from (i) Apaf-1$^{+/-}$ or (j) Apaf-1$^{-/-}$ mice.

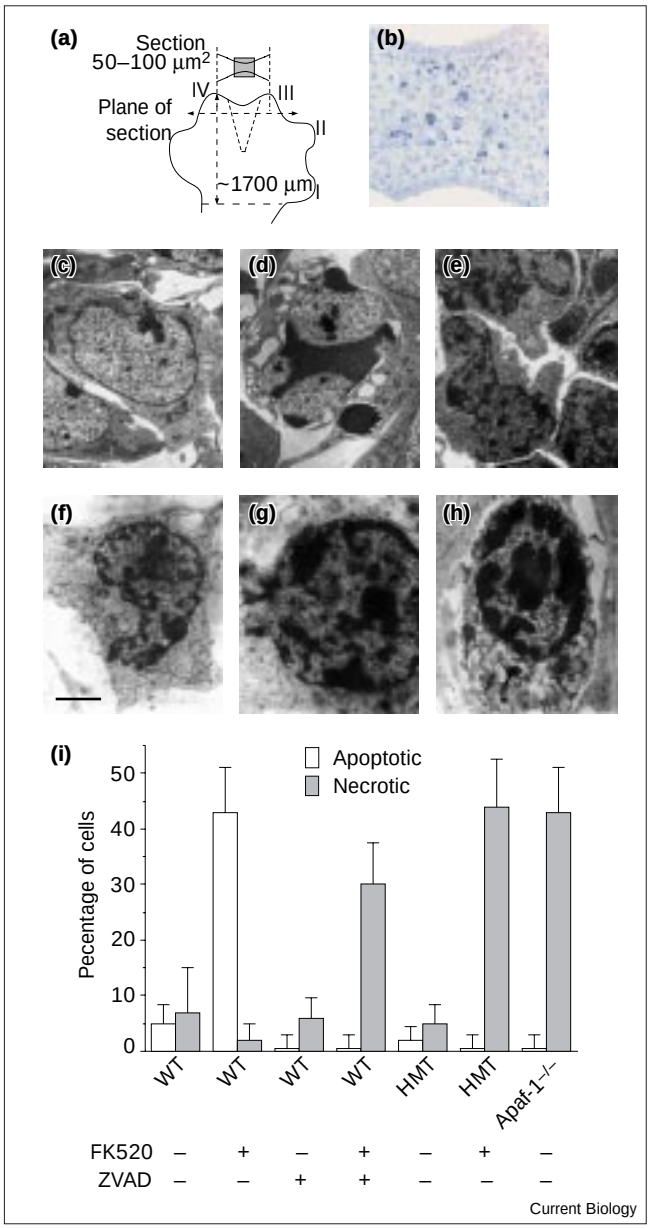
generally more frequent in the central areas of the interdigital spaces seen in transverse sections, indicating that the reagents used did not induce indiscriminate 'toxic' cell death. Rather, when 15-O-desmethyl-FK520 was added, the spatial distribution of cell death, whether apoptotic in the absence or necrotic in the presence of ZVAD-fmk, seemed to follow the normal developmental pattern. Thus, short-term *in vitro* incubation of wild-type limb buds in the presence of 15-O-desmethyl-FK520 led to increased interdigital apoptotic cell death. Apoptosis was prevented by coincubation with a caspase inhibitor which led to the appearance of necrotic cells, however. Although these experiments allowed necrotic cells in interdigital spaces to be easily identified, the use of drugs raises questions about specificity.

To reduce the use of drugs, we first resorted to Hammertoe mice which, because of an undefined mutation, show no or very limited cell death in interdigital spaces 2–4 of the hind limb buds [2]. As was shown before [2], when the hind limb buds of Hammertoe mice were incubated for 6 hours in medium, interdigital spaces 2–4 showed few if any TUNEL$^+$ cells (Figure 1g; interdigital space 1 had the expected percentage of TUNEL$^+$ cells, data not shown). By EM, there were 2% (1–6%) apoptotic cells and 5% (2–9%) necrotic cells. Upon limb-bud incubation for 6 hours in the presence of 15-O-desmethyl-FK520, sections showed cell-free gaps, with still few TUNEL$^+$ (Figure 1h) and condensed cells; by EM there were 0% (0–5%) apoptotic cells but, now, 44% (36–53%) of cells had a necrotic morphotype (Figure 2f). Thus, in Hammertoe mice, addition of 15-O-desmethyl-FK520 led to cell death, but the genetic defect in interdigital spaces 2–4 prevented the appearance of apoptotic cells while allowing that of necrotic cells. We next measured the kinetics of this necrotic cell death. Whereas a control group incubated for 8 hours *in vitro* without 15-O-desmethyl-FK520 showed 2% (1–7%) necrotic cells, after 2, 4, 6 and 8 hours incubation in the presence of 15-O-desmethyl-FK520, there were by EM 6% (3–9%), 54% (47–61%), 50% (43–57%) and 80% (75–85%) necrotic cells, respectively, and no apoptotic cells at any time. In transverse sections, necrotic cells appeared at 2–4 hours in the central area of interdigital spaces, then more necrotic cells appeared peripherally while most of the cells in the centre disappeared, indicating an asynchrony of signalling and/or time course of cell death across the interdigital space in these conditions. While showing that necrotic cells can appear in marked proportions in the absence of ZVAD-fmk treatment, these experiments relied on the uncharacterised mutation of Hammertoe mice and on the drug 15-O-desmethyl-FK520.

To avoid using drugs or any *in vitro* incubation, we used Apaf-1$^{-/-}$ mice. Apaf-1 is required within apoptosomes in a mitochondrial context for downstream caspase activation [9]. Control Apaf-1$^{+/-}$ limb buds showed an apparently normal proportion of apoptotic cells (Figure 1i). In limb buds from Apaf-1$^{-/-}$ mice, activation of caspase-3 is prevented [10,11]. Nevertheless, in Apaf-1$^{-/-}$ mice, interdigital cells must ultimately die, as digit separation, although delayed, is completed in surviving neonates ([11] and our unpublished data). Indeed, in these mice, many necrotic cells were observed, which showed mottled chromatin condensation, nuclear membrane detachment and rupture, dilated mitochondria and cytoplasmic vacuoles, and sometimes external membrane rupture (Figure 2g,h). Various intermediate aspects could also be observed (data not shown), which ranged from apparently intact cells that had a mottled nucleus, to cells that had a mottled nucleus and

Figure 2

Interdigital cell death viewed by EM. To reduce the area investigated to one that would be small enough to be screened by EM, we used transverse instead of axial limb-bud sections. To increase reproducibility, we usually examined sections at the same depth, that is, about 90 μm below the apical ectodermal ridge (AER). Most of the obvious gaps, linked presumably to cell death and removal, reside somewhat further away from the AER (see Figure 1). Transverse sections were performed across interdigital space 3. The digits are numbered I–V. (a) Schematic representation of the plane of transverse section. (b) Freshly fixed Unna-blue-stained transverse semithin section of a mouse interdigital space. The Unna-blue stained apoptotic cells appear condensed. (c–e) Wild-type NMRI limb buds, incubated for 6 h in (c) medium alone, showing apparently viable cells, or in the presence of (d) 15-O-desmethyl-FK520, showing apoptotic cells, or (e) 15-O-desmethyl-FK520 and ZVAD-fmk, showing necrotic cells. (f–h) Examples of necrotic cells from (f) limb buds of Hammertoe mutant mice incubated in the presence of 15-O-desmethyl-FK520 for 4 h, and (g,h) freshly fixed Apaf-1$^{-/-}$ limb buds. The scale bar in (f) represents (g,h) 1 μm, (d,e) 1.7 μm and (c,f) 2.3 μm. (i) Percentages of cells with an apoptotic or a necrotic morphotype in various normal, experimental or mutant mouse situations. Limb buds from wild-type (WT), Hammertoe (HMT) or Apaf-1$^{-/-}$ mice were incubated for 6 h with or without 15-O-desmethyl-FK520 (FK520) and/or ZVAD-fmk (ZVAD). To count cells with the respective morphotypes in a transverse interdigital section, 10–20 low-power (× 1600) EM photographs were assembled, showing a total of 150–200 cells, of which the proportions of apoptotic or necrotic cells were established and are given as percentages ($p = 0.05$).



gross membrane alterations. Altogether, in these sections of freshly-fixed Apaf-1$^{-/-}$ limb buds, there were 43% (36–50%) necrotic cells, little indication at any stage of TUNEL^{+} cells (Figure 1j), and 0% (0–5%) apoptotic cells as judged by EM. Thus, necrotic cells were the only cells with a recognisable cell-death morphotype in an interdigital web which was being developmentally removed. Whereas apoptosis includes phagocytosis (see also Figure 2d), here even heavily altered necrotic cells appeared not to be within phagocytes (Figure 2e–h). This did not prevent removal of the interdigital web (although this removal was delayed), and did not lead to obvious local signs of inflammation, but this absence might also be related to local anatomical, possibly vascular, constraints.

Are necrotic cells also present in normal wild-type interdigital spaces not subjected to drug treatment? By EM screening, we found, in normal freshly-fixed interdigital spaces, 6% (2–10%) necrotic cells in addition to the 5% (2–9%) apoptotic cells mentioned above. Thus, during normal development some cells might die with a necrotic morphotype. Alternatively, under normal conditions, at least some dying cells might temporarily present a mottled nucleus, before a caspase-mediated shift to the apoptotic morphotype. Although some cells with only a mottled nucleus might not progress to more marked lesions and to cell death, most of these cells must be dying, as they show within a disappearing web a morphological continuum from only a mottled nucleus to marked necrotic lesions inconsistent with cell survival. There have been reports of caspase-independent mammalian cell death (although not during development) with a morphotype similar to the one described here, obtained in the presence of ZVAD-fmk [12–14], or in cells from caspase-3 knock-out mice [15], or by ligation of major histocompatibility complex (MHC) class I molecules [16]. A similar morphotype has been described during insect metamorphosis [17] and for the nuclear patterns generated by apoptosis-inducing factor (AIF) [18]. Necrotic cell death would be missed when cell death is assessed using apoptosis-specific methods such as the widely used TUNEL technique. Also, by light microscopy, necrotic cells are less conspicuous than apoptotic cells. Therefore, in some pathological, experimental and perhaps natural situations, the extent of non-apoptotic cell death, thus of cell death as a whole, might be underestimated.

Thus, cell death can occur in interdigital cells under conditions when conventional caspases cannot operate, perhaps the first such direct indication in a mammalian developmental context. Caspases, if involved, would have to be unconventional enough for their activity not to lead to DNA fragmentation, not to be blocked by ZVAD-fmk, and not to be abolished in Apaf-1$^{-/-}$ mice. We favour the simpler interpretation that both the necrotic morphotype and this mechanism of cell death occur in the absence of caspase activity. As shown in the Hammertoe mutant mouse, this process leads to cell death in a matter of hours. Our data with 15-O-desmethyl-FK520 suggest that the same BMP receptors might be at play in caspase-dependent and caspase-independent cell death, consistent with the observation that, apparently, the same interdigital cell populations die in wild-type and in Apaf-1$^{-/-}$ mice, and to the same extent in surviving Apaf-1$^{-/-}$ neonates. Caspase-dependent apoptosis and caspase-independent necrosis can originate from the same receptor complex [19,20]. The caspase-dependent and caspase-independent mechanisms might diverge downstream of a common (perhaps mitochondrial) caspase-independent cell-death trigger, which might require AIF or cytochrome c but not APAF-1, as Apaf-1$^{-/-}$ mice show necrosis.

The loss of interdigital cells is a paradigm of cell death during development. In this model, necrosis occurred when 15-O-desmethyl-FK520 was added and apoptosis was prevented from occurring by the application of ZVAD-fmk, or by the Hammertoe mutation. In wild-type mice not treated with drugs, some interdigital cells were observed to be necrotic, although reservations might be raised because of the small percentage of these cells. Nevertheless, this mode of cell death was clearly seen in Apaf-1$^{-/-}$ mice where, within a regressing interdigital web, the only recognisable cell death morphotype was that of necrosis. That necrosis was seen in diverse situations indicates that it is not linked to the toxic effect of a particular condition. In interdigital webs of Apaf-1$^{-/-}$ mice, caspase-independent necrosis occurs as part of development in a programmed, non-accidental manner. Caspase-independent necrosis might account in part for the relatively limited extent of abnormalities seen in some caspase-null mutant mice. These results strongly suggest the existence, in interdigital cells, of a programmed caspase-independent cascade of events leading to necrosis, underlying the more classical caspase-dependent cascade leading to apoptosis.

Supplementary material

Supplementary material including additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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Supplementary material

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Supplementary materials and methods

Mice

Pregnant NMRI females (received from CERJ, Le Genest Saint Isle, France) were sacrificed by cervical dislocation at embryonic day (E) 13.5. For Hammertoe mice (generous gift from B. Robert, Institut Pasteur, Paris; maintained at the CIML on a C57Bl/6 background), females with a vaginal plug in the morning after mating were considered at day 0.5 of gestation and sacrificed at E14.5. Apaf-1$^{-/-}$ embryos were obtained from Apaf-1$^{+/-}$ F1 crosses and sacrificed at E14.5. The Apaf-1$^{+/-}$ mice were initially derived from 129Sv × NMRI crosses, backcrossed up to F5 into a C57Bl/6 genetic background.

Limb buds

Limb buds were cut and either fixed in 4% paraformaldehyde ('freshly fixed') or incubated usually for 6 h at 37°C either with 0.1% DMSO as a solvent control ('medium only'), or with 100 μM ZVAD-fmk (Bachem, Voisins-le-Bretonneux, France) and/or with 100 μM 15-O-desmethyl-FK520 (a generous gift from Sandoz /Novartis through M. Schulz), and then fixed. For frozen sections, limbs were rinsed in PBS, fixed in 4% paraformaldehyde at 4°C overnight and then soaked in 20% sucrose in PBS at 4°C for 4 h. They were embedded in OCT compound (Tissue Tek) and immediately frozen at –80°C. Frozen limbs were cut using a cryomicrotome (Jung CM3000, Leica).

Sections

Sections (7 or 8 μm) were transferred onto precoated slides (Esco, Superfrost Plus). Slides were dried at room temperature and stored at –80°C. For TUNEL assays, *In situ* Apoptosis Detection kit (TACS™ 2 TdT/blue label, TREVIGEN, Sigma, L'Isle d'Abeau-Chesnes, France) was used according to the manufacturer's instructions, except that no permeabilization step was required. Observations were with a Leitz DM microscope and pictures were taken with a Leica MPS52 camera (Leica). For semithin sections and transmission electron microscopy, limb buds were fixed in glutaraldehyde/paraformaldehyde (2% + 2%) overnight at 4°C and postfixed in 1% osmium tetroxide in PBS for 1 h. Samples were dehydrated in a graded series of ethanol then in acetone (30 min), soaked in acetone/Epon (50:50) for 30 min, embedded in pure Epon overnight and mounted between two plastic slides polymerised first at 37°C overnight then at 60°C for 2 days. Semithin sections (1 μm) were cut with an ultramicrotome (RMC) and stained with Unna blue. For electron microscopy, ultrathin sections (80/90 nm) were mounted on single hole copper grids, coated with butvar and stained with 2% uranyl acetate for 30 min, followed by lead citrate for 8 min. Sections were examined with a Zeiss EM 912 electron microscope (Zeiss) at 100 kV.