# Types of programmed cell death: two variants expressed by neonatal murine hepatocytes

Sasaki K, Sonoda Y, Kumano I and Suda M

Department of Anatomy, Kawasaki Medical School, Matsushima 577, Kurashiki, 701-0192 Japan

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Correspondence

Prof. Kazunobu Sasaki

Department of Anatomy

Kawasaki Medical School

577 Matsushima, Kurashiki, 701-0192 JAPAN

TEL: 086-462-1111 ext 86319

FAX: 086-462-1199

E-mail: kazus@med.kawasaki-m.ac.jp

Abbreviations used in this paper

| | |
|---|---|
| dUTP | 2'-deoxyuridin 5'-triphosphate |
| ER | endoplasmic reticulum |
| TdT | terminal deoxynucleotide transferase |
| TUNEL | terminal transferase-mediated dUTP nick end labeling |

**Abstract**

Neonatal livers examined by the TUNEL method contained numerous positive cells. Although the majority of dying cells are either hematopoietic cells including erythroids and granulocytes or macrophages, a few hepatocytes were also positive. As for the ultrastructural features of these dying hepatocytes, two different types: type I and II, could be identified. The early features of type I appeared in the cytoplasm, which was characterized by dilated rough endoplasmic reticulum, and the cell fragments displayed a round, foamy appearance. Type II was characterized by nuclear compaction and margination of heterochromatin resulting in formation of sharply circumscribed masses, followed by condensation of the cytoplasm. The cell death of type I, characterized by the formation of massive vacuolization of the endoplasmic reticulum, corresponds to cytoplasmic type degeneration or non-apoptotic death, while that of type II corresponds to nuclear type cell death or classical apoptotic death. In the two types of programmed cell death, the incidence of non-apoptotic cell death was much higher than that of classical apoptosis in neonatal murine hepatocytes.

## Introduction

Hematopoiesis in the mouse fetal liver starts at 10 days of gestation, after which the liver becomes the major site of hematopoiesis during the middle period of embryogenesis. This is followed by hematopoietic eclipse after 15 days of gestation [Sasaki and Sonoda, 2000]. Since most hematopoietic cells disappear from the liver shortly after birth, neonatal livers are in a transitional stage between fetal hematopoietic and adult liver function. Fetal liver hematopoiesis is predominantly erythropoietic, and not only hepatocytes but also macrophages are involved in forming the hematopoietic foci [Sasaki and Iwatsuki, 1997; Sonoda et al., 2001]. The fetal liver is known to be the major site for erythropoietin production [Zanjani et al., 1977], and erythropoietin plays important roles in maintaining a homeostatic balance of erythropoiesis and apoptosis in hepatic erythroid foci [Yu et al., 1993]. Although liver stromal cells are a major part of the structural support of the hematopoietic microenvironment, little information is available on hepatocyte morphology with respect to the hematopoietic involution of early postnatal livers. In midway in our investigation, we recognized that some hepatocytes underwent cell death in neonatal livers, and, in addition to the well-known apoptosis originally noted by Wyllie et al. (1980), another route in the hepatocyte death processes could be identified. As far as is known, this report is the first to morphologically show programmed cell death in neonatal hepatocytes.

## Materials and Methods

Livers from 30 postnatal ICR mice at 0, 2, 3, 4, 5, 6, 7, 8 and 9 days after birth were used in this study.

*Cell death labeling procedures*

The TUNEL assay was performed using the 'Apoptosis in situ Detection Kit' (Wako Pure Chemicals) according to the manufacturer's instructions. Briefly, livers were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4, 4℃) for 4 hrs, and the tissues were embedded in paraffin. Serial sections, 3 μm thick, were dewaxed and treated with proteinase K for 5 min. The 3'-hydroxyl ends of DNA were labeled with fluorescein-dUTP by terminal deoxynucleotidyl transferase (TdT) at 37℃ for 10 min. The labeled DNA was detected by incubation with peroxidase-conjugated anti-fluorescein antibody at 37 ℃ for 10 min, followed by incubation with diaminobenzidine. The sections were counterstained with eosin. As positive controls, sections were incubated with DNase I for 15 min after protease incubation, and for negative controls, sections were incubated with TdT buffer that did not contain the enzyme.

*Epon-embedded and re-embedded procedures*

The removed livers were cut into small blocks and immediately immersed in 4% paraformaldehyde with 5% glutaraldehyde in 0.1 M cacodylate buffer (Karnovsky's fluid) for 3 hrs. After fixation, the tissue blocks were postfixed in 2% osmium tetroxide for 2 hrs. Following washing in distilled water, they were dehydrated in graded ethanols and embedded in Epon 812. Serial semithin sections, 1 μm thick, were cut with a diamond knife and stained in 1% toluidine blue. After examination and photography by light microscopy, sections selected for electron microscopy were re-embedded. Selected sections were covered with a size #0 gelatin capsule containing Epon 812 and incubated at 60℃ overnight for polymerization. When the gelatin

capsules were quickly removed from the slides, the sections were successfully attached to the side of the capsules. Then the semithin sections were trimmed and cut at a thickness of 90 nm with a Leica Ultracut S and a diamond knife. Ultrathin sections were mounted on formvar film-coated single hole copper grids, and, after double staining in uranyl acetate and lead citrate, observations were carried out with a JEM-2000 EXII electron microscope operating at 80kV.

## Results

### A. Cell deaths in neonatal livers

*Cellular constitution of the neonatal livers*

In early neonatal livers from 0 to five days after birth, the majority of hepatocytes were closely associated with each other, gathering in clusters, and sinusoids meandered between hepatocyte clusters (Fig.1a). The hepatocytes partially became arranged radially with respect to central veins, but lobulation and the regular organization of the hepatocytes into one-cell-thick cell plates were not achieved. The hepatocytes were large polyhedral cells, and the majority had a single round nucleus with one to two nucleoli and abundant cytoplasm containing several small lipid droplets. Clusters of hepatocytes occasionally contained round cell fragments having a characteristic foamy appearance between two and four days after birth (Fig.1b). Sections of the livers contained a considerable number of hematopoietic foci until five days after birth (Fig.1a). The foci were diffusely observed either within hepatocyte clusters or in the perisinusoidal space between sinusoid endothelium and the hepatocytes, and consisted of hematopoietic cells and a few macrophages. In the solitary hematopoietic foci, various blood cell lineages, including erythroid, myeloid

Fig.1

and lymphocyte lineages, could be identified and macrophages often had large phagocytotic inclusions. After five days, the hematopoietic foci decreased in number, and both lobulation and the development of hepatic cell plates occurred by nine days of age.

*TUNEL-positive cells*

Neonatal livers examined by the TUNEL method contained numerous positive cells. The majority of positive cells were found in hematopoietic foci, and TUNEL stained the nuclei of not only hematopoietic cells (Fig.2a) but also macrophages (Fig.2b). In addition, between two and five days of age, a few TUNEL-positive hepatocytes were also observed (Fig.2c). Compared with hematopoietic cells, the number of TUNEL-positive hepatocytes was small.

Fig.2

*Hepatocytes undergoing cell death*

Semithin plastic sections of neonatal livers stained by toluidine blue occasionally contained hepatocytes which showed morphological features taken as early manifestations of cell death under light microscopy (Fig.3). These early processes of hepatocyte death appeared to take one of the following two forms: cytoplasmic involvement, designated as type I cell death; or the nuclear involvement, designated as type II cell death. Under light microscopy, type I cell death in hepatocytes was characterized by changes not only in cell outline, the polyhedral profile of intact cells to a circular outline, but also in cytoplasmic density, from dark to light (Fig.3a). On the other hand, type II cell death was characterized by cellular and nuclear shrinkage and chromatin margination and condensation along the nuclear envelope (Fig.3b), although the cytoplasm in type II cell death had the same density as intact hepatocyte cytoplasm.

Fig.3

148 For identifying hepatocytes undergoing programmed cell death under light microscopy,
149 there were several advantages to observations in Epon-embedded sections over ones in
150 paraffin sections. Epon-embedded sections showed that neonatal hepatocytes
151 contained numerous round lipid droplets in their cytoplasm, permitting a distinction
152 between lipid droplets and vacuoles. In contrast, in paraffin sections, due to the
153 dissolution of lipid material from the cytoplasm during the embedding procedure, the
154 cytoplasm of intact hepatocytes also had a foamy appearance, and distinctions between
155 intact hepatocytes and hepatocytes undergoing type I cell death were therefore difficult
156 to draw.
157
158 **B. Ultrastructural features of type I and II hepatocyte death**
159 The intact hepatocytes of neonatal livers were polyhedral in profile, and the
160 electron-dense cytoplasm was filled with abundant cell organelles including numerous
161 mitochondria and numerous long flattened sacs of rough endoplasmic reticulum (ER)
162 arranged parallel to each other (Fig.4a). Mitochondria were round to oval in shape and
163 around 0.6 μm in size. The cytoplasm also contained a few peroxisomes, approximately
164 0.3 μm in diameter, with a crystalline core. The ultrastructural features of the early
165 stages of type I cell death appeared in the cytoplasm. Compared with neighboring
166 intact hepatocytes, the cytoplasm became electron-lucent, characterized by small round
167 profiles of dilated rough ER (Fig.4a,b). The profiles of the dilated rough ER were round
168 and were 0.2 – 0.3 μm in diameter. Intact hepatocytes had numerous microvilli
169 projecting into bile canaliculi, but there were few microvilli on the canalicular surface
170 of the dying hepatocytes (Fig.4c). The nuclei had a round to ovoid contour with a
171 smooth outline. Mitochondria showed a slight increase in size, being larger than 1 μm,
172 but the mitochondrial membrane and cristae appeared intact. Due to marked

Fig.4

expansion and fusion of parallel cisternae of the dilated ER, the cytoplasm of the hepatocytes became entirely filled with numerous large and clear vacuoles, showing a foamy appearance (Fig.5a,b). The vacuoles showed considerable variation in size and shape. Perinuclear cisternae of the foamy hepatocytes also became dilated, and the nuclear outline became slightly irregular (Fig.5c). Mitochondria, lipid droplets and peroxisomes were diffusely scattered throughout the cytoplasm. In hepatocytes at the advanced stages of type I cell death, the nucleus disappeared, and ER sometimes clustered, forming large spherical accumulations (Fig.6a). Mitochondria as well as lipid droplets could be found surrounding the accumulations (Fig.6b). Cell fragments at the final stage displayed a massively vacuolated ER (Fig.7a,b), but specialized cell contact, such as the presence of desmosomes, could still be recognized (Fig.7b). Cell fragments, less than five μm in diameter, contained contracted mitochondria, peroxisomes and lipid droplets, and all ribosomes had disappeared (Fig. 7c,d). Mitochondria appeared slightly contracted, but their internal structures remained intact. Loss of hepatocytic junctional attachments resulted in the release of the cell fragments into perisinusoidal spaces.

Fig.5

Fig.6

Fig.7

The early features of the other cell death process, type II, on the other hand, mainly appeared in the nucleus. The nucleus became irregularly shaped, being characterized by compaction and margination of heterochromatin, resulting in the formation of sharply circumscribed masses (Fig.8). The cytoplasmic volume was markedly decreased, and the cisternae of the rough ER were slightly distended in parallel array (Fig.8a). The constricted cytoplasm contained numerous lipid droplets and mitochondria, although mitochondrial profiles varied widely in size (Fig.8b,c). Dying hepatocytes detached from the cell plates and moved into the sinusoidal lumen

Fig.8

198 (Fig.8a). Debris from dying cells was phagocytosed by hepatocytes as well as by
199 sinusoidal macrophages. At the ultrastructural level, autophagocytosis of either
200 mitochondria or endoplasmic reticulum could not be identified in hepatocytes
201 undergoing type I or II cell death.
202
203 In early neonatal livers, there was a much higher incidence of type I death
204 than type II, and classical apoptosis was minimally expressed in programmed cell death
205 in murine hepatocytes. Hepatocyte death showed a rapid decrease in degree after five
206 days of age. The ultrastructural features of the two types of hepatocyte death processes
207 are schematically summarized in Figure 9.

Fig.9

208
209
210 **Discussion**
211 The embryonic liver is a major but transient hematopoietic organ, and one of
212 the most interesting problems posed by the hepatic phase of hematopoiesis is the decline
213 in activity. Since liver hematopoiesis in the mouse embryo reaches a peak at 13 days
214 of gestation, to decline as early as 15 days of gestation, the livers of both late gestational
215 fetuses and early neonates correspond to the involution stage of hematopoiesis [Sasaki
216 and Sonoda, 2000]. The fetal mouse liver has been reported to exhibit apoptosis of
217 hematopoietic cell lines [Yu et al., 1993], and our results confirm the morphological
218 evidence that hepatocytes also undergo developmental cell death in early neonates.
219 The neonatal liver actually contains significant amounts of hepatocyte debris derived
220 from two different processes of degradation, which could not be identified by paraffin
221 sections stained by a routine method.
222

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in morphological and biochemical changes in dying cells [Wyllie et al., 1980]. In the process of apoptosis, the first characteristic is condensation of chromatin around the nuclear membrane, followed by fragmentation of the nucleus, cell blebbing and the formation of membrane-bound apoptotic bodies [Cohen, 1993; Bonanno et al., 2000]. In addition to the above classical apoptosis, several different mechanisms or processes of programmed cell death have been noted in fetal organogenesis [Lockshin and Zakeri, 2002]. In reviewing the literature, Clarke [1990] proposed that there are three main types of developmental cell death in developing tissues; type I - apoptosis, type II - autophagic degeneration, and type III - non-lysosomal vesiculate degradation. Type III degradation is composed of two subtypes; type 3A- non-lysosomal disintegration and type 3B- a cytoplasmic type of degeneration. Type 3B cell death involves the dilatation of rough endoplasmic reticulum, and there is neither early condensation of the nucleus nor detectable fragmentation of the cell. Therefore, the majority of hepatocyte cell deaths occurring in the neonatal mouse livers are partly consistent with Clarke's type 3B cell death. This cytoplasmic type of degeneration shares several features with necrotic cell death, i.e., dilatation of the endoplasmic reticulum and late karyolysis [Clarke, 1990]. At present, several routes to physiological and pathological pathways in programmed cell death have been determined [Liou et al, 2003], and links among the pathways appear to be very interesting subjects for further investigation [Lockshin and Zakeri, 2002].

Our results showed that hepatocytes underwent programmed cell death following two distinct processes, and that the major process of this death was characterized by cytoplasmic vacuolization mainly caused by dilatation of the

endoplasmic reticulum. When hepatocytes were treated with either a low dose of cycloheximide in combination with brefeldin A or tunicamycin, an ultrastructural study revealed that massive vacuolization of the ER was induced, followed by apoptosis, mainly due to their toxic and metabolic insults that perturb ER function [Zinszner et al., 1998; Häcki et al., 2000]. The ultrastructural features of cell organelles after doses of agents that perturb ER function appeared to be very similar to those of type I hepatocyte death in the neonatal mouse. Zakeri et al. [1995] reported that there are at least two major types of physiological cell death; type I being classical apoptosis and type II being characterized by vacuolization of the cytoplasm with delayed collapse of the nucleus. Vacuole formation was commonly seen both in Zakeri's type II cell death and in the type I cell death of neonatal hepatocytes, but many of the cytoplasmic vacuoles in Zakeri's observation were of lysosomal rather than endoplasmic origin. Therefore, the cell organelles involved in the processes of cytoplasmic cell death could not belong to a single group of cell organelles but must belong to several different ones.

It is widely accepted that gross swelling of mitochondria in which flocculent and granular densities occur appears as the earliest ultrastructural marker of cells undergoing necrosis [Walker et al, 1988; Padanilam, 2003]. Perhaps the most notable cell organelle that triggers programmed cell death is the mitochondrion, which contains a special 'cell suicide protein' in the intermembrane space. The opening of transition pores in the inner membrane leads to mitochondrial swelling which precedes nuclear deformity [Petit et al, 1996, Skulachev, 1996]. As shown in our results, the mitochondria in hepatocytes undergoing both type I or II cell death showed slight changes only in their size. Although further quantitative investigations are necessary to pin down probable mitochondrial involvement in the programmed death of neonatal

hepatocytes, morphological changes in ER in type I and in the nucleus in type II appeared more prominent than those in mitochondria. Häcki et al.[2000] reported the presence of a ER and mitochondria crosstalk pathway for apoptosis induction caused by perturbation of ER function. Taken together, our findings imply that more attention should be given not only to mitochondria, but also to ER involvement in the programmed cell death of neonatal hepatocytes.

Fetal hepatocytes have been assumed to exert their influence on liver erythropoiesis directly through the production of erythropoietin [Zanjani et al., 1981; Flake et al., 1987; Koury and Bondurant, 1990; Aiuti et al., 1998]. As mentioned earlier, hematopoiesis reached a peak at 13 days of gestation and then showed a decline as early as 15 days of gestation [Sasaki and Sonoda, 2000]. However, the appearance of foamy cell fragments of type I cell death between three and five days after birth did not parallel the changes in hematopoietic activity. Detailed analyses of the significance of the two independent types of hepatocyte death are the subject of further investigation in association with functional changes in the ER in hepatocytes before and after birth.

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**Figure legends**

Figure 1. A three-day-old liver. Toluidine blue-stained 1 μm section.

a) A low-power micrograph.

Small clusters of hematopoietic cells (arrows) are found diffusely among hepatocytes and in perisinusoidal spaces between hepatocytes and twisting sinusoid (*). The arrangement of hepatocytes is irregular, and the organization of the hepatocytes into the cell plates has not been fully achieved. IV: interlobular venule. X 500.

b) High-power micrographs of the framed areas of a).

A foamy cell profile (arrow) can be easily recognized on a semithin section under light microscopy. X 760.

Figure 2. Four-day-old livers stained by TUNEL and eosin. Paraffin sections. X1,500.

a) A TUNEL-positive hematopoietic cell.

The arrow indicates a positive polymorphonuclear leukocyte in a hematopoietic focus (HF). S: sinusoid.

b) A TUNEL-positive macrophage in a hematopoietic focus.

A macrophage (arrow) centrally situated in a hematopoietic focus is TUNEL-positive. Asterisks indicate erythroblasts.

c) A TUNEL-positive hepatocyte.

A positive hepatocyte nucleus (arrow) appears small in size when compared with the pale nuclei (arrowheads) of intact hepatocytes.

Figure 3. Light micrographs of hepatocytes showing early signs of cell death.

1μm Epon sections stained by toluidine blue. 3-day-old liver. X960.

a) Type I cell death.

The arrow indicates a hepatocyte at the early stage of cell death. The hepatocyte, round in profile, represents a paler cytoplasmic matrix.

b) Type II cell death.

The hepatocyte in the circle has a nucleus showing marginated and sharply delineated masses of uniformly dense chromatin. The cytoplasmic volume is markedly decreased but the matrix preserves some density.

Figure 4. Type I cell death - an early stage. Four days of age. Bar: 1 μm.

a) Intact hepatocytes and a dying hepatocyte.

Intact hepatocytes (IH) are generally polyhedral in shape, and the cytoplasm appears electron dense. Compared to the normal cytoplasm, the dying hepatocyte (DH) is characterized by an electron-lucent cytoplasm, but the nucleus conserves a normal image.

b) A micrograph of the framed area in a).

The electron-lucent cytoplasm contains numerous dilated cisternae of the endoplasmic reticulum with round profiles (arrows). Some of mitochondria (M) show a slight increase in size when compared with those in neighboring intact hepatocytes, but the mitochondrial membrane and cristae appear unaffected. N: nucleus.

c) A bile canaliculus of the framed area in b) .

Three junctional complexes (J) can be seen around the bile canaliculus. Numerous microvilli (arrows) project from intact hepatocytes into the canaliculus, but there are few microvilli (arrowhead) on the dying hepatocyte.

Figure 5. Foamy hepatocytes of type I cell death. Bar: 1μm.

a) Neonatal liver at three days of age.

The nucleus (N) is roughly round but irregularly contoured. Due to the highly dilated cisternae of the endoplasmic reticulum, the cytoplasm has a characteristic foamy appearance. H: intact hepatocytes, S: sinusoid.

b) Neonatal liver at 4 days of age.

Dilated cisternae fuse to form large vacuoles which fully occupy the cytoplasm. The nucleus (N) appears intact.

c) A high-power micrograph of the framed area in b).

Asterisks indicate dilated perinuclear cisternae. Narrow septa of the cytoplasm contain mitochondria, and cytoplasmic ribosomes have disappeared with vacuole formation.

Figure 6. Advanced stage of type I cell death. Three days of age. Bar: 1μm.

a) A low-power micrograph.

Hepatocyte fragments of advanced stage often lacked a nucleus and contained large accumulations of smooth endoplasmic reticulum (ER).

b) A high-power micrograph of the framed area in a).

Mitochondria (M) and lipid droplets (arrows) are scattered around the ER accumulation. Almost all ribosomes have disappeared, and the cytoplasmic matrix looks empty. ER accumulation is frequently observed.

Figure 7. Final stage of type I cell death.

a) A foamy fragment. 4-day-old liver. Bar: 1 μm.

The fragment contains lipid droplet accumulations (LD). H: intact hepatocyte.

b) A high-power micrograph of the framed area (b) in a). Bar: 0.2 μm.

A desmosome (between two arrows) can still be recognized between the fragment and a neighboring hepatocyte.

c) A high-power micrograph of the framed area (c) in a). Bar: 0.5 μm.

The arrows indicate mitochondria, some of which show a slight contraction, and mitochondrial cristae appear intact.

d) Peroxisomes in hepatocyte fragments of the final stage. Bar: 0.5 μm.

A few peroxisomes (arrows) which have crystalline inclusions could be seen among lipid droplets (L) and mitochondria (M).

Figure 8. Type II cell death. Bar: 1μm.

a) An early apoptotic hepatocyte (AH). 3-day-old liver.

Compaction and margination of heterochromatin are prominent. The hepatocyte has detached from the liver cell plates, and is now located in the sinusoidal lumen (S). This electron micrograph was taken of an ultrathin section from the reembedded semithin section shown in Figure 3b. Three days of age.

b) An apoptotic hepatocyte (AH). 7-day-old liver.

The hepatocyte shows a nuclear deformity, and the chromatin shows typical condensation. The narrow cytoplasm is full of lipid droplets.

c) A high-power micrograph of the framed area in b).

Mitochondria (*) show considerable variations in size but the mitochondrial membrane and cristae remain unaltered.. N: Nucleus, L: Lipid droplet.

Figure 9. A schematic illustration showing the two death processes in hepatocytes in

458 neonatal livers.

459 The massive formation of vacuoles of ER origin in type I and the nuclear changes
460 in type II are especially emphasized. As shown by the large arrows, there is a
461 much higher incidence of type I cell death than type II (dotted small arrow) in
462 neonatal hepatocytes.