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2 **Types of programmed cell death: two variants**
3 **expressed by neonatal murine hepatocytes**

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25

26 Abbreviations used in this paper

27 dUTP 2'-deoxyuridin 5'-triphosphate

28 ER endoplasmic reticulum

29 TdT terminal deoxynucleotide transferase

30 TUNEL terminal transferase-mediated dUTP nick end labeling

31

32 **Abstract**

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

47

48 **Introduction**

49 Hematopoiesis in the mouse fetal liver starts at 10 days of gestation, after
50 which the liver becomes the major site of hematopoiesis during the middle period of
51 embryogenesis. This is followed by hematopoietic eclipse after 15 days of gestation
52 [Sasaki and Sonoda, 2000]. Since most hematopoietic cells disappear from the liver
53 shortly after birth, neonatal livers are in a transitional stage between fetal hematopoietic
54 and adult liver function. Fetal liver hematopoiesis is predominantly erythropoietic,
55 and not only hepatocytes but also macrophages are involved in forming the
56 hematopoietic foci [Sasaki and Iwatsuki, 1997; Sonoda et al., 2001]. The fetal liver
57 is known to be the major site for erythropoietin production [Zanjani et al., 1977], and
58 erythropoietin plays important roles in maintaining a homeostatic balance of
59 erythropoiesis and apoptosis in hepatic erythroid foci [Yu et al., 1993]. Although
60 liver stromal cells are a major part of the structural support of the hematopoietic
61 microenvironment, little information is available on hepatocyte morphology with
62 respect to the hematopoietic involution of early postnatal livers. In midway in our
63 investigation, we recognized that some hepatocytes underwent cell death in neonatal
64 livers, and, in addition to the well-known apoptosis originally noted by Wyllie et al.
65 (1980), another route in the hepatocyte death processes could be identified. As far as
66 is known, this report is the first to morphologically show programmed cell death in
67 neonatal hepatocytes.

68

69

70 **Materials and Methods**

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

73

74 *Cell death labeling procedures*

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

87

88 *Epon-embedded and re-embedded procedures*

89 The removed livers were cut into small blocks and immediately immersed in
90 4% paraformaldehyde with 5% glutaraldehyde in 0.1 M cacodylate buffer (Karnovsky's
91 fluid) for 3 hrs. After fixation, the tissue blocks were postfixed in 2% osmium
92 tetroxide for 2 hrs. Following washing in distilled water, they were dehydrated in
93 graded ethanols and embedded in Epon 812. Serial semithin sections, 1 µm thick,
94 were cut with a diamond knife and stained in 1% toluidine blue. After examination
95 and photography by light microscopy, sections selected for electron microscopy were
96 re-embedded. Selected sections were covered with a size #0 gelatin capsule containing
97 Epon 812 and incubated at 60°C overnight for polymerization. When the gelatin

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

104

105

106 **Results**

107 **A. Cell deaths in neonatal livers**

108 *Cellular constitution of the neonatal livers*

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

Fig.1

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

127

128 *TUNEL-positive cells*

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

135

136 *Hepatocytes undergoing cell death*

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

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

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

Fig.5

Fig.6

Fig.7

189
190 The early features of the other cell death process, type II, on the other hand,
191 mainly appeared in the nucleus. The nucleus became irregularly shaped, being
192 characterized by compaction and margination of heterochromatin, resulting in the
193 formation of sharply circumscribed masses (Fig.8). The cytoplasmic volume was
194 markedly decreased, and the cisternae of the rough ER were slightly distended in
195 parallel array (Fig.8a). The constricted cytoplasm contained numerous lipid droplets
196 and mitochondria, although mitochondrial profiles varied widely in size (Fig.8b,c).
197 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

223 Two distinct modes of cell death, apoptosis and necrosis, can be distinguished
224 based on differences in morphological and biochemical changes in dying cells [Wyllie
225 et al., 1980]. In the process of apoptosis, the first characteristic is condensation of
226 chromatin around the nuclear membrane, followed by fragmentation of the nucleus, cell
227 blebbing and the formation of membrane-bound apoptotic bodies [Cohen, 1993;
228 Bonanno et al., 2000]. In addition to the above classical apoptosis, several different
229 mechanisms or processes of programmed cell death have been noted in fetal
230 organogenesis [Lockshin and Zakeri, 2002]. In reviewing the literature, Clarke
231 [1990] proposed that there are three main types of developmental cell death in
232 developing tissues; type I - apoptosis, type II - autophagic degeneration, and type III -
233 non-lysosomal vesiculate degradation. Type III degradation is composed of two
234 subtypes; type 3A- non-lysosomal disintegration and type 3B- a cytoplasmic type of
235 degeneration. Type 3B cell death involves the dilatation of rough endoplasmic
236 reticulum, and there is neither early condensation of the nucleus nor detectable
237 fragmentation of the cell. Therefore, the majority of hepatocyte cell deaths occurring
238 in the neonatal mouse livers are partly consistent with Clarke's type 3B cell death.
239 This cytoplasmic type of degeneration shares several features with necrotic cell death,
240 i.e., dilatation of the endoplasmic reticulum and late karyolysis [Clarke, 1990]. At
241 present, several routes to physiological and pathological pathways in programmed cell
242 death have been determined [Liou et al, 2003], and links among the pathways appear to
243 be very interesting subjects for further investigation [Lockshin and Zakeri, 2002].

244

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

248 endoplasmic reticulum. When hepatocytes were treated with either a low dose of
249 cycloheximide in combination with brefeldin A or tunicamycin, an ultrastructural study
250 revealed that massive vacuolization of the ER was induced, followed by apoptosis,
251 mainly due to their toxic and metabolic insults that perturb ER function [Zinszner et al.,
252 1998; Häcki et al., 2000]. The ultrastructural features of cell organelles after doses of
253 agents that perturb ER function appeared to be very similar to those of type I hepatocyte
254 death in the neonatal mouse. Zakeri et al. [1995] reported that there are at least two
255 major types of physiological cell death; type I being classical apoptosis and type II
256 being characterized by vacuolization of the cytoplasm with delayed collapse of the
257 nucleus. Vacuole formation was commonly seen both in Zakeri's type II cell death
258 and in the type I cell death of neonatal hepatocytes, but many of the cytoplasmic
259 vacuoles in Zakeri's observation were of lysosomal rather than endoplasmic origin.
260 Therefore, the cell organelles involved in the processes of cytoplasmic cell death could
261 not belong to a single group of cell organelles but must belong to several different ones.

262

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

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

279

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

290

291

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349

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356

357

358 **Figure legends**

359 Figure 1. A three-day-old liver. Toluidine blue-stained 1 μ m section.

360 a) A low-power micrograph.

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

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

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

369

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

372 a) A TUNEL-positive hematopoietic cell.

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

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

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

378 c) A TUNEL-positive hepatocyte.

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

381

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

383 1 μm Epon sections stained by toluidine blue. 3-day-old liver. X960.

384 a) Type I cell death.

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

387 b) Type II cell death.

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

391

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

393 a) Intact hepatocytes and a dying hepatocyte.

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

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

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

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

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

408

409 Figure 5. Foamy hepatocytes of type I cell death. Bar: 1 μ m.

410 a) Neonatal liver at three days of age.

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

414 b) Neonatal liver at 4 days of age.

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

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

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

421

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

423 a) A low-power micrograph.

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

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

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

430

431 Figure 7. Final stage of type I cell death.

432 a) A foamy fragment. 4-day-old liver. Bar: 1 μ m.

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

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

435 A desmosome (between two arrows) can still be recognized between the fragment

436 and a neighboring hepatocyte.

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

438 The arrows indicate mitochondria, some of which show a slight contraction, and

439 mitochondrial cristae appear intact.

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

441 A few peroxisomes (arrows) which have crystalline inclusions could be seen

442 among lipid droplets (L) and mitochondria (M).

443

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

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

446 Compaction and margination of heterochromatin are prominent. The hepatocyte

447 has detached from the liver cell plates, and is now located in the sinusoidal lumen

448 (S). This electron micrograph was taken of an ultrathin section from the

449 reembedded semithin section shown in Figure 3b. Three days of age.

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

451 The hepatocyte shows a nuclear deformity, and the chromatin shows typical

452 condensation. The narrow cytoplasm is full of lipid droplets.

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

454 Mitochondria (*) show considerable variations in size but the mitochondrial

455 membrane and cristae remain unaltered.. N: Nucleus, L: Lipid droplet.

456

457 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.