Apoptosis in Rabbit Haemorrhagic Disease


Dept. of Veterinary Public Health and Laboratory Animal Medicine, College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, 103 Seodun-dong, Kionsun-Gu, Suwon 441–744, Korea

Summary

Rabbit haemorrhagic disease virus (RHDV) causes an acute hepatitis and disseminated intravascular coagulation. Six rabbits were inoculated experimentally with RHDV to investigate any potential relationship between infection and apoptosis in the liver. Two rabbits were killed at 12 h post-inoculation (PI) and two at 24 h PI. The remaining two rabbits died at 30 h and 31 h PI. Immunohistochemical labelling for RHDV antigen-positive cells, TUNEL assay for apoptotic cells, and DNA analysis were performed on samples of liver. The four rabbits that died or were killed 24–31 h PI had acute hepatitis with infiltration of heterophils and necrotic hepatocytes. RHDV antigen-positive cells and apoptotic cells appeared in the centriacinar areas at 12 h PI; subsequently they spread to periacinar areas and increased in number, but the viral antigen-positive cells outnumbered apoptotic cells. At 24–31 h PI, few apoptotic cells were recognized in the areas infiltrated with lymphocytes and heterophils. The results suggested an association between RHDV infection and apoptosis of hepatocytes.

Introduction

The family Caliciviridae contains the single genus, Calicivirus. Members include human pathogens such as Norwalk virus, and animal pathogens such as primate and feline calicivirus and rabbit haemorrhagic disease virus (RHDV). Their genome consists of c. 7·5 kb of positive-sense single-stranded (ss) RNA and is characterized by two to three open reading frames. Caliciviruses possess a single capsid protein of about 60 kDa (Prasad et al., 1994a,b). Rabbit haemorrhagic disease (RHD) is an acute calicivirus infection, occurring only in rabbits and initially recognized in China (Liu et al., 1984). The causative agent, RHDV, has a predilection for the cytoplasm of hepatocytes (Ohlinger et al., 1990; Meyers et al., 1991; Park et al., 1992a,b). Rabbits inoculated experimentally with RHDV die within 3 to 4 days. Acute necrotic hepatitis, disseminated intravascular coagulation (DIC), and haemorrhage in various organs are typical findings at necropsy (Fuchs and Weissenbock, 1992; Plassiart et al., 1992; Ueda et al., 1992).

Apoptosis plays an important role in the cytopathogenicity of some viruses (Kawanishi, 1993; Laurent-Crawford et al., 1993; Hinshaw et al., 1994; Ubol et al., 1996). The relationship between viruses and apoptosis is a complex one, partly because all viruses are faced with the need first to replicate within a receptive intracellular environment, then to spread to other cells or tissues, and finally to seek out new hosts to continue the chain of propagation (Collins, 1995; Razvi and Welch, 1995).

Using rabbits inoculated with RHDV, we investigated in the study described here a potential relationship between RHDV infection and apoptosis of hepatocytes by examining the chronological changes in the distribution of RHDV antigen-positive cells and apoptotic cells.

Materials and Methods

Virus

A liver homogenate was prepared from a rabbit that had died of experimentally induced RHDV infection. The purified virus from the liver homogenate had the following characteristics: density
of 1·31 to 1·33 g/cm$^3$ by CsCl density gradient centrifugation, resistance to lipid-soluble solvent, hollow depressions on the surface of virus particles, and a major molecular mass of 63 kDa (Park et al., 1991).

**Animals**

Eight New Zealand White rabbits weighing 3·3 to 4·0 kg were purchased from Daehan Ltd, Seoul, Korea. These rabbits were negative for antibody to RHDV in a haemagglutination-inhibition assay. All the animals were acclimatized for 1 week and maintained throughout the study in a room with a temperature of $19 \pm 1^\circ$C, humidity of $55 \pm 5\%$, and a 12-h light-dark cycle ($220 \pm 80$ lux). The animals were housed in pairs in stainless steel wire mesh cages ($100 \times 100 \times 35$ cm) and given free access to commercial pellets (Samyang Ltd, Seoul, Korea) and water. During acclimatization all the rabbits remained free of clinical signs of RHD. Two of the rabbits were used as uninfected controls, but the remaining six animals each received an intramuscular injection of 0·5 ml of liver homogenate (haemagglutination titre, $2^{10}$) prepared from a rabbit that had died from RHD. Pairs of rabbits were killed at 12 and 24 h post-inoculation (PI), by cervical artery section under pentobarbital (40 mg/kg of body weight, intravenously) anaesthesia. The remaining two infected rabbits were subjected to post-mortem examination immediately after their death at 30–31 h PI.

**Samples and Methods of Examination**

Sample of liver, lung and kidney were fixed in neutral buffered formalin and embedded in paraffin wax for histopathological examination. To investigate apoptosis in relation to the presence of RHDV antigen, serial sections ($3\mu$m) of the liver were prepared for staining with haematoxylin and eosin (HE), assay by terminal deoxy-nucleotidyl transferase-mediated digoxigenin-dUTP nick end labelling (TUNEL), and immunohistochemical labelling. The numbers of RHDV antigen-positive cells and apoptotic cells were counted in eight lobules in two sections for each rabbit. A portion of liver was taken and rapidly frozen in liquid nitrogen for DNA isolation and electrophoresis.

**Histopathology**

Sections ($3\mu$m) were examined after preparation and staining with HE by routine methods.

**TUNEL Assay**

Liver sections were examined with the ApopTag kit (Oncor, Gaithersburg, MD, USA). After dewaxing and rehydration, sections were treated with proteinase K (20 $\mu$g/ml) for 15 min at room temperature. Endogenous peroxidase was blocked by $\text{H}_2\text{O}_2$ 2% in methanol for 5 min. Digoxigenin-dUTP end labelled DNA was detected with antidigoxigenin-peroxidase antibody, followed by peroxidase detection with 0·05% diaminobenzidine (DAB) containing $\text{H}_2\text{O}_2$ 0·02%. The tissue sections were counterstained with methyl green.

**Immunohistochemical Detection of RHDV Antigen**

Liver sections were stained by the direct avidin–biotin-peroxidase complex method (Park et al., 1992a). The sections were dewaxed and rehydrated, and endogenous peroxidase was blocked by $\text{H}_2\text{O}_2$ 0·3% in methanol for 10 min. To block non-specific binding, the sections were incubated with 10% normal rabbit serum containing bovine serum albumin (BSA) 1% and skimmed milk 5% for 1 h. The sections were incubated with biotinylated rabbit anti-RHDV IgG at 4$^\circ$C overnight. This was followed by washing in three changes of PBS (0·12 M NaCl, 0·01 M Na$_2$HPO$_4$, 0·04 M K$_2$HPO$_4$, 0·05% Tween 20) for 30 min each, and then incubation in avidin and biotinylated horseradish peroxidase macro-molecular complex (ABC) solution (Vector Laboratories, Burlingame, CA, USA) for 100 min. After three further 15-min washes in PBS, a 5-min incubation in 0·05% DAB containing $\text{H}_2\text{O}_2$ 0·05% was used to identify peroxidase activity. The sections were counterstained with haematoxylin.

**DNA Isolation and Agarose Gel Electrophoresis**

DNA was isolated from liver tissue frozen in liquid nitrogen, according to the method described by Sambrook et al. (1989). The frozen liver was homogenized in DNA extraction buffer (10 mM Tris-HCl, pH 8·0, 100 mM EDTA, sodium dodecyl sulphate 0·5%, RNAse A 100 $\mu$g/ml) and incubated for 16 h at 50$^\circ$C in the presence of proteinase K 100 $\mu$g/ml. The DNA was extracted with phenol/ chloroform and precipitated in ethanol. Pellets were resuspended in $T_{od}E_{od}$ (10 mM Tris-HCl, pH 8·0; 1 mM EDTA), and the DNA concentration was determined from the absorbance at 260 nm. Each DNA sample was electrophoresed through a 2·0% agarose gel containing ethidium bromide 1 mg/ml. DNA bands were “visualized” with an ultraviolet transilluminator and photographed.
Results

Clinical and Post-mortem Observations

The infected rabbits became lethargic at 12 h PI and those allowed to die naturally did so at 30–31 h PI. In rabbits that died, or were killed at 24–31 h PI, the liver was pale brown and friable, with a distinct lobular pattern. Pulmonary haemorrhage was also observed in the two rabbits that died at 30–31 h PI.

Histopathological Findings

At 12 h PI, necrotic hepatocytes were occasionally observed in the centriacinar areas and heterophils were slightly increased in the hepatic sinusoids. Moderate numbers of heterophils in the lungs and congestion in the kidneys were observed.

At 24 h PI, marked degeneration and necrosis of hepatocytes were observed. The lesions were confluent in the centriacinar areas and occasionally in the periacinar areas (Fig. 1). Some hepatocytes were undergoing lysis by heterophil migration into the cytoplasm, and the cytoplasmic membrane was difficult to recognize. Heterophil numbers were also moderately increased in the spleen and the lungs. Numerous thrombi were evident in the capillaries of the kidneys and lungs.

In the rabbits that died at 30–31 h PI, degeneration and necrosis of hepatocytes were observed focally and diffusely in the lobules of the liver. The necrotic hepatocytes were shrunken and occasionally infiltrated with heterophils; they also showed marked nuclear changes such as karyorrhexis, karyopyknosis and karyolysis. Heterophil infiltration was evident in clumped and individual necrotic hepatocytes. Frequently, small eosinophilic globules were present in the sinusoids.

Immunohistochemical Detection of RHDV Antigen

Viral antigen was stained deep brown and was either distributed diffusely in the cytoplasm or concentrated as fine granules at the periphery of the hepatocytes. The number of RHDV antigen-positive hepatocytes increased with time post-inoculation. At 12 h PI, the viral antigen was detected in some hepatocytes, mainly in the centriacinar areas. At 24 h PI, it was detected in many hepatocytes in the centriacinar areas and in a few in the periacinar areas (Fig. 2). In the rabbits that died at 30–31 h PI, many hepatocytes containing RHDV antigen were observed throughout the lobules but remained more prominent in the centriacinar areas. However, few RHDV antigen-positive cells were identified in the areas in which necrotic hepatocytes were infiltrated with heterophils.

TUNEL Assay

The number of apoptotic hepatocytes increased with time post-inoculation (Fig. 3a–c). At 12 h PI, a few were observed in the centriacinar areas (Fig.
3a). At 24 h PI, many apoptotic hepatocytes were detected in the periphery of lobules (Fig. 3b). In the rabbits that died at 30–31 h PI, such cells were numerous (Fig. 3c). Many apoptotic hepatocytes had fragmented nuclei. Few apoptotic cells were present in areas infiltrated by heterophils.

**RHDV Antigen and Apoptotic Cell Counts**

Serial sections of the liver treated immunohistochemically or by the TUNEL method were used to count RHDV antigen-positive cells and apoptotic cells in liver lobules (Table 1). The numbers of the RHDV antigen-positive cells were 20.8, 48.8 and 156.4 at 12 h, 24 h and 30–31 h PI, respectively. The numbers of the apoptotic cells were, however, 0.4, 7.4, 28.2, and 117.0 at 0 h (controls), 12 h, 24 h and 30–31 h PI, respectively. Thus, RHDV antigen-positive cells outnumbered apoptotic cells at all stages PI.

**DNA Isolation and Agarose Gel Electrophoresis**

The hepatocytes containing fragmented nuclei were RHDV antigen-positive. A faint DNA ladder pattern typical of apoptosis in normal rabbits was observed in the livers of the control rabbits. A similar faint DNA staining pattern was also observed in infected rabbits at 12 h PI. However, a marked increase in the intensity of the DNA ladder pattern was observed at 24 and 30–31 h PI (Fig. 4).
Discussion

The histopathological characteristics of RHD have been described previously (Nowotny et al., 1990; Carrasco et al., 1991; Alexandrov et al., 1992; Stoerckle-Berger et al., 1992). The initial change in the liver is ballooning degeneration of hepatocytes, progressing to necrosis, with infiltration of heterophils into necrotic areas. Such observations are consistent with a lytic viral infection causing hepatocyte disruption with subsequent release of viral antigen and heterophil infiltration (Park et al., 1995, 1997). In this study, the degeneration and necrosis of hepatocytes in the rabbits inoculated with RHDV were initially observed in the centriacinar areas and the changes seemed to spread progressively to the entire lobule. Lytic change in hepatocytes became evident at 24 h and 30–31 h PI, probably due to infiltration of heterophils into hepatocytes.

RHDV antigen-positive cells in the liver were distributed in a pattern similar to that of the histopathological lesions, initially in the centriacinar area and later in the periacinar area (Park et al., 1995). Apoptosis of hepatocytes was confirmed by the TUNEL technique and by identification of low-molecular-weight DNA fragmentation patterns on agarose gels. Increasing in number with time post-inoculation, apoptotic hepatocytes were observed throughout the liver lobules. The RHDV antigen-positive cells outnumbered apoptotic cells at all stages post-inoculation. These results strongly suggest an association between viral infection and apoptosis of hepatocytes. Apoptotic cells and viral antigen-positive cells were not observed in the areas infiltrated by heterophils.

Viruses have developed strategies to inhibit or stimulate apoptosis, depending on the particular virus-host interaction (Hinshaw et al., 1994; Collins, 1995; Razvi and Welch, 1995). The premature induction of apoptosis may be either detrimental to the virus by disrupting the viral replicative life

<table>
<thead>
<tr>
<th>Cells</th>
<th>0 (controls)</th>
<th>12</th>
<th>24</th>
<th>30–31</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHDV-positive</td>
<td>0</td>
<td>20·8±2·8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48·8±2·4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>156·4±18·0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Apoptotic</td>
<td>0·4±0·5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7·4±1·8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28·2±4·3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>117·0±16·2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The numbers of RHDV antigen-positive cells and apoptotic cells were counted in eight lobules in two sections for each rabbit. 

<sup>a,b,c</sup> Means in each row with different superscripts are significantly different at <i>P</i><0·05.
cycle, or advantageous by facilitating viral dissemination and secondary infection of phagocytes or other immune cells (Collins, 1993; Razvi and Welch, 1995). Viral hepatitis is a characteristic of RHD. It may be due either to direct hepatocytic damage in response to viral infection, or to infiltration of immune cells. Hepatitis in rabbits infected with RHDV is induced by virus rather than by an immune response (Park et al., 1995).

In this study, apoptotic changes in hepatocytes were typical of RHD and the results suggested that apoptosis of hepatocytes was induced by RHDV. Studies are now needed on identification of an apoptosis-induction gene in RHD viral hepatitis.

Acknowledgment

This work was supported by the Brain Korea 21 Project.

References


[Received, May 17th, 1999]
[Accepted, March 13th, 2000]