In vivo apoptosis of hepatocytes in guinea pigs infected with Leptospira interrogans serovar icterohaemorrhagiae

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Abstract

To investigate the contribution of the previously demonstrated in vitro apoptosis to the pathogenesis of leptospirosis, guinea pigs were infected with Leptospira interrogans serovar icterohaemorrhagiae strain Verdun and sequentially killed to collect target organs involved in the natural history of the disease (liver, kidneys, lungs, spleen and heart). The combination of histopathological procedures and a specific TUNEL assay showed a significant Leptospira-induced programmed cell death of hepatocytes with a peak at 48 h post inoculation. Hepatocyte nuclei showed morphological changes including fragmented and condensed nuclei. This phenomenon occurred early in the course of the disease at a time where infecting leptospires were present at a low density between the liver parenchyma cells.

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1. Introduction

Leptospirosis is a world-wide veterinary and human public health problem [1], caused by spirochetes belonging to the genus Leptospira [2–4]. Human leptospirosis is highly variable in its clinical expression. The course of the disease ranges from mild flu-like symptoms to rapidly fatal forms, and the most severe cases of human leptospirosis are mainly caused by Leptospira interrogans serovar icterohaemorrhagiae isolates.

Little is known regarding the pathogenesis of leptospirosis. Laboratory rodents (young guinea pigs or hamsters) are susceptible to a limited number of virulent Leptospira strains. In addition, pathogenic leptospires are cultured in media not resembling conditions in vivo, and virulence is gradually lost after some passages [1]. After skin or mucosal penetration, leptospires reach the bloodstream where avirulent ones are cleared rapidly [1]. Conversely, after a bacteremic stage, virulent leptospires reach and colonise the target tissues of the host organism [1]. However, the pathophysiological cellular mechanisms of leptospirosis are unclear. Recently, we have demonstrated in vitro that a virulent strain of L. interrogans
killed macrophages through apoptosis and was able to invade Vero cells [5]. Apoptosis is a mode of cell death that occurs under normal physiological conditions. In addition to physiological stimuli, bacteria have been reported to induce apoptosis in host cells [6] such as in vitro cultured macrophages [7–11]. Furthermore, there is increasing evidence for the involvement of apoptosis in bacterial pathogenesis as demonstrated in vivo in different target cells [12–15].

The purpose of this study was to investigate the induction of apoptosis using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-dependent nick-end labelling) assay, in target tissues involved in the different stages of leptospirosis (liver, kidneys, lungs, spleen and heart). Guinea pigs were experimentally infected with a virulent strain of \( L. \) interrogans and sequentially killed in a kinetic study. Complementary data on the distribution of leptospires within the target tissues and the nature of the corresponding histological lesions are also reported.

2. Materials and methods

2.1. Bacterial strains and cultivation

The virulent \( L. \) interrogans Verdun, serovar icterohaemorrhagiae, was from the Reference Collection of the Pasteur Institute in Paris, France. As previously defined [5], cultures (in EMJH medium) derived from cardiac puncture of infected animals were used exclusively for experimental infections. Two groups of eight specific-pathogen-free Dunkin-Hartley ICO:DH guinea pigs, 10–12 days old (weighing less than 150 g), were lethally infected subcutaneously with \( 10^8 \) leptospirosis (liver, kidneys, lungs, spleen and heart). Guinea pigs were experimentally infected with a virulent strain of \( L. \) interrogans and sequentially killed in a kinetic study. Complementary data on the distribution of leptospires within the target tissues and the nature of the corresponding histological lesions are also reported.

2.2. Immunological reagents

New Zealand White rabbits weighing approximately 3–4 kg each were used for the immunisations with live \( L. \) interrogans strains according to reference methods [1]. Agglutination titres of at least 1:12800 were obtained and sera were aliquoted and stored at \(-20^\circ C\) until use.

2.3. Indirect immunofluorescence staining

After tissue rehydration, a 150-μl aliquot of the leptospiral rabbit antiserum diluted 1 to 100 in PBS containing 0.5% bovine serum albumin (BSA) was applied to each wet slide for 45 min at \( 37^\circ C \) before being washed in PBS. Then, slides were incubated under the same conditions with fluorescein-labelled goat anti-rabbit immunoglobulin G \( F(ab')_2 \) fragment (Boehringer, Mannheim, Germany). Finally, slides were examined with a Leitz DMRBE epifluorescence microscope.

2.4. In situ apoptosis analysis

Cell apoptosis was investigated in different tissue sections with the TUNEL technique used in the in situ cell death detection kit (Boehringer). Briefly, apoptosis-induced DNA strand breaks can be identified by labelling free 3’OH termini with fluorescein-labelled nucleotides using the terminal deoxynucleotidyl transferase (TdT). This allows the detection of DNA fragmentation by fluorescence microscopy directly after the TUNEL reaction. Subsequently, an anti-fluorescein antibody Fab fragment conjugated with horseradish peroxidase was added and counterstaining was performed for histological examinations.

Deparaffinised and rehydrated tissue sections were subjected to proteinase K digestion (500 μg ml\(^{-1}\) in 10 mM Tris-HCl pH 7.5 at \(37^\circ C\) for 30 min). After PBS washing, a blocking solution (0.3% \( H_2O_2 \) in methanol) was applied for 30 min at room temperature. Slides were incubated for 3 min at 4°C in a 0.3% Triton X-100 in PBS solution to permeate cells. Some improvements were performed in order to ensure sensitive results: (i) the specimens were exposed for 2 h at \( 37^\circ C \) in a moist chamber to the TUNEL labelling mix containing 0.5 U μl\(^{-1}\) calf thymus TdT and nucleotide mix containing fluorescein-dUTP. (ii) In comparative experiments, the respective efficiencies in DNA labelling of the TdT (Boehringer) and the ApopTag TdT enzymes (Oncor, Gaithersburg, Millford).
MD) were evaluated. Coverslips were rinsed in PBS and incubated with an anti-fluorescein antibody (Fab fragments from sheep antibody conjugated with horseradish peroxidase; 7.5 U ml$^{-1}$) at 37°C for 45 min. Then, 3,3′-diaminobenzidine (Boehringer) was used as substrate for 20 min at room temperature. Finally, TUNEL-labelled tissue sections were counterstained before microscopy, using the periodic acid Schiff technique. Each slide was first screened at low magnification (×400) to detect brown nuclei corresponding to apoptotic cells with subsequent observation at a higher magnification (×1000) using a video amplifier (CCD-IRIS, Sony, Japan). Photomicrographs were taken with a 400 ASA reversed film (Eastman Kodak Company, Rochester, NY) using an Orthomat E system.

2.5. Histological examination

Each section used for in situ apoptosis analysis was compared to an adjacent specimen stained with hematoxylin and eosin.

Fig. 1. Evidence of apoptosis in the liver of a guinea pig infected with a virulent strain of Leptospira. Slides were treated with the TUNEL protocol. A: Non-infected control showing an isolated apoptotic nucleus (green fluorescence) in the parenchyma (white arrow). B: 48 h after infection, a higher proportion of apoptotic nuclei was observed (white arrows). Magnification ×400.
2.6. Statistical analysis

Randomly selected fields in each slide were examined and apoptosis was expressed as the mean number of apoptotic cells per 100 cells, in three independent assays, for each organ, and the standard deviation was determined.

3. Results

Significant labelling differences were seen according to the enzyme employed, as the TdT enzyme possessed a higher labelling capacity than ApopTag TdT (data not shown). Consequently, TdT DNA (Boehringer) labelling was used throughout the experiments. At 72 h post inoculation (p.i.), macroscopic findings in autopsied guinea pigs consisted of jaundice, scattered petechia, enlargement of the spleen, discoloured liver, swollen kidneys and pulmonary haemorrhages. Apoptosis-induced DNA strand breaks were detected using epifluorescence microscopy. Typical morphological changes (condensed chromatin and fragmented nuclei) were also identified. We first demonstrated that *L. interrogans* serovar icterohaemorrhagiae was able to induce a significant increase of apoptosis in the liver compared with the non-infected control (Fig. 1). In contrast, the intensity of apoptosis in other target organs was not significantly different from the corresponding basal controls. To characterise cells dying by apoptosis, the previous TUNEL protocol was followed by a horseradish peroxidase detection method, and counterstaining using the periodic acid Schiff technique. As previously demonstrated, a significant increase of apoptosis was detected in the infected liver compared with the corresponding non-infected sample. Considering their morphological characteristics, cells undergoing apoptosis were identified as hepatocytes (Fig. 2). When compared to the physiological hepatocytic apoptosis in controls (0.1%), a significant increase in the number of apoptotic hepatocytes in infected guinea pigs was observed (Fig. 3). This began 8 h p.i. (0.25%; infected to basal ratio = 2:1) with a maximum at 48 h. p.i (5.3%; infected to basal ratio = 53:1). Then, the percentage of apoptotic hepatocytes progressively decreased (1% at 72 h p.i.; infected to basal ratio = 10:1) to return to the physiological level by 96 p.i. (0.1%). The other organs did not show a significant increase in apoptotic cells whatever the time period during the course of infection (data not shown).

As shown in Table 1, leptospires stained by indirect immunofluorescence were detectable (magnification ×400) in the liver as soon as 24 h p.i. at a low level (≤1 leptospira per field) with a heterogeneous

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Fig. 2. Identification of apoptotic cells in a liver section of a guinea pig infected with a virulent strain of *Leptospira* (48 h p.i.). Slides were treated with the TUNEL protocol and counterstained with periodic acid Schiff method. Brown nuclei are representative of apoptotic hepatocytes (black arrows). Note the typical fragmented and condensed chromatin. Magnification ×750.
distribution in the parenchyma. The infectivity profile exhibited a gradual increase with a maximum of more than 100 leptospires per field by 96 h, with a homogeneous distribution in the intercellular space between the hepatocytes, producing a continuous fluorescent network (Fig. 4). Some leptospires were also visible in the lumen of the vessels. Comparatively, the spleen showed the same time-dependent infectivity profile. However, by 96 h p.i., this rate remained below 100 leptospires per field (Table 1). In the heart, leptospires were detected later (48 h p.i.) and their density progressively increased without reaching the maximum level of the bacteraemic stage observed in the liver. In lungs and kidneys,

Table 1
Distribution of leptospires (magnification ×400) and histological lesions in selected target tissues of experimentally infected guinea pigs with a virulent strain of Leptospira

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time (h) after experimental infection</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
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<tbody>
<tr>
<td>Liver</td>
<td>Distribution of leptospires&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>xx</td>
<td>xxx</td>
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<td>Kidneys</td>
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<td>Heart</td>
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<td>Lungs</td>
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<td>Spleen</td>
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<td>—</td>
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<td>x</td>
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<td>x</td>
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<td>x</td>
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<tr>
<td>Liver</td>
<td>Histological lesions&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>—</td>
<td>—</td>
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<td>Kidneys</td>
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<td>Heart</td>
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<tr>
<td>Lungs</td>
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<td>Spleen</td>
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</table>

<sup>a</sup> —, absence of leptospires; x, 1 leptospira per field; xx, >1–10 leptospires per field; xxx, >10–100 leptospires per field; xxxx, >100 leptospires per field.

<sup>b</sup> ▲, haemorrhagic focus; ▲ ▲, haemorrhagic area; □, capillary dilatation; □ ■, oedema, weak congestion; □ □ □, oedema, intense congestion; ◻, absence of inflammatory infiltrate; ◻ ◻, discrete inflammatory infiltrate.
leptospires were detected 24 h p.i. but did not exceed 100 leptospires per microscopic field even by 96 h p.i.

Histological examination of selected tissues is summarised in Table 1. Globally, a phase of tissue oedema was immediately followed by the development of an inflammatory infiltrate 24-48 h p.i. Haemorrhagic foci were evidenced first in the lungs from 24 h p.i. In the liver, in addition to apoptosis in hepatocytes, inflammatory foci with lymphocytes infiltrating portal tracts appeared by 48 h p.i. and were followed by the development of haemorrhagic areas; apoptotic cells were not identified within these lesions.

4. Discussion

Several histopathological studies of leptospirosis have been reported previously, and these mainly focused on the description of lesions observed in the liver and the kidneys [16–18]. In experimental infection of Syrian hamsters, pathogenic leptospires were observed in the liver, in large number in the intercellular spaces, without systematic alterations of the surrounding hepatocytes [19]. Indeed, the occasional presence of leptospires within the cytoplasm of parenchymal cells, such as hepatocytes or tubular renal cells, was reported during the acute stage of the disease [17–19]. Although the hypothesis of a toxin has been suggested [20], its role in acute leptospirosis has never been demonstrated. All in all, the nature and the mechanisms of the cellular lesions of the main target organs in leptospirosis remain unknown.

Pathogen-induced apoptosis can occur either after internalisation of bacteria or entry of bacterial components into the target cell or by binding to a host cell receptor [6]. Pathogen-induced apoptosis of hepatocytes has rarely been reported excepted in the case of *Listeria monocytogenes* which induced apoptosis of hepatocytes in experimentally infected CB-17 mice [13].

According to our results, the peak of apoptosis within the liver was observed 48 h p.i. at a time when virulent leptospires are present at low concentration (<10 bacteria per microscopic field). In addition to detection by the TUNEL method, cells undergoing apoptosis were characterised by morphological changes and nuclear degradation. Apoptosis of hepatocytes seems to be an early event in the infection process during leptospirosis. Considering the limited inflammatory reaction at this stage of the hepatic involvement, it could be hypothesised that *L. interrogans*-induced apoptosis would favour the bacteria by inhibiting the molecular signalling of targeted hepatocytes, and cause a transient decrease in the recruitment of inflammatory cells. This process would allow the leptospires to colonise the liver efficiently. Necrosis arises later and massively from 72 h p.i. concurrently with the proliferation of macrophages and neutrophils. Lastly, this earliness and limitation of apoptosis to hepatocytes is an additional argument for the importance of the hepatic stage for local multiplication and subsequent dissemina-
tion of virulent leptospires to other target organs. This is in agreement with the natural history of the human disease where the acute forms of leptospirosis evolving to a multisystemic infection occur preferentially in patients with icterus and hepatic deficiency [21,22].

In a previous in vitro study [5], we showed that apoptosis of a macrophage-like cell line by L. interrogans was initiated after entry of the pathogen into the host cell. Conversely, Vero cells (a renal fibroblast cell line) were invaded by virulent leptospires but apoptosis did not occur [5]. Concurrently, apoptosis was not in evidence in kidneys in our in vivo experimental model. Lastly, considering the localisation of leptospires in the intercellular spaces of the liver parenchyma, it appears that virulent leptospires do not need to invade hepatocytes to induce their programmed cell death.

In conclusion, we have demonstrated that L. interrogans serovar icterohaemorrhagiae is able to induce apoptosis in guinea pig hepatocytes. This phenomenon occurs early in the course of the disease and could contribute to an efficient colonisation of the liver and to the subsequent diffusion of leptospires in the animal. The results presented here provide new insight into the pathogenesis of leptospirosis which remains globally misunderstood. Further investigations should focus on the mechanisms of induced apoptosis in hepatocytes in order to identify the signal transduction pathways.

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