Apoptotic cell death of vascular endothelial cells and renal tubular cells in the generalized Shwartzman reaction


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Abstract

The participation of apoptotic cell death in the generalized Shwartzman reaction was examined. The generalized Shwartzman reaction was induced in mice by two consecutive injections of lipopolysaccharide. Vascular endothelial cells in various organs of those mice were stained positively by the in situ specific labeling of fragmented DNA. Renal tubules were also stained focally. It was suggested that apoptotic cell death might participate in the development of vascular endothelial cell damage and acute tubular necrosis in the generalized Shwartzman reaction. Simultaneous administration of anti-γ-interferon antibody in the preparative injection of lipopolysaccharide completely blocked apoptosis of vascular endothelial cells. Priming with recombinant γ-interferon instead of lipopolysaccharide could produce apoptosis of vascular endothelial cells. It was suggested that γ-interferon might play a critical role on sensitization of endothelial cells for apoptosis.

Keywords: Lipopolysaccharide; Apoptosis; Shwartzman reaction; Interferon; Fragmented DNA

1. Introduction

Bacterial lipopolysaccharide (LPS) is present on the outer membranes of all Gram-negative bacteria and causes the systemic inflammatory response syndrome, endotoxic shock and disseminated intravascular coagulation (DIC). The generalized Shwartzman reaction (GSR) is a potentially lethal shock reaction, which can be induced by two consecutive injections of LPS (called a preparative injection and a provocative one, respectively) into animals at an interval of 24 h [1-4]. GSR is characterized by vascular occlusion, hemorrhage, perivascular accumulation of leukocytes, and necrosis [1,2], and it is known as an experimental DIC model [5]. It has been reported that GSR and DIC are due to systemic injuries and death of vascular endothelial cells [2,5]. Cell death can generally proceed via necrosis or apoptosis (programmed cell death). Necrosis is characterized by the formation of tubular lesions in the plasma membrane, whereas apoptosis causes cell death in a different way from necrosis morphologically and biochemically [6-8]. The common characteristic mechanism of apoptosis is known to be DNA fragmentation and morphological lesions, such as condensation.
and fragmentation of the nucleus and cytoplasm [6–8].

Recently we have reported that in vivo administration of LPS to mice induces DNA fragmentation of thymocytes, and that the target cells are CD4+8+ immature cortical thymocytes [9–11]. The administration of LPS and D-galactosamine induces massive hepatocyte apoptosis in the liver [12,13]. Thus, it became clear that cell death in response to LPS might be caused by apoptosis. It is likely that apoptotic cell death participates in systemic injuries of vascular endothelial cells in response to LPS. GSR provides a useful experimental system for characterization of injury of vascular endothelial cells by LPS. In the present study, we examined if and how apoptosis was involved in systemic injuries and death of vascular endothelial cells in response to LPS using GSR-induced mice.

2. Materials and methods

2.1. Animal

Male BALB/c mice were purchased from SLC (Hamamatsu, Japan), and used at 8–10 weeks of age.

2.2. Reagents

Rat monoclonal antibodies neutralizing murine tumor necrosis factor α (TNF-α) and γ-interferon (IFN-γ) were purchased from UB Inc., Lake Placid, NY, USA. Murine recombinant IFN-γ, interleukin (IL)-2, TNF-α, and hamster monoclonal antibodies neutralizing IL-1β and IL-2 were obtained from Genzyme Co., Cambridge, MA, USA. They were used as indicated by the manufacturer.

Fig. 1. Induction of apoptosis of vascular endothelial cells in the livers of GSR-induced mice. GSR was induced in four BALB/c mice by two consecutive injections of LPS; 7 h after the injection the livers were subjected to nick end labeling specific for fragmented DNA. Typical positive nuclei of vascular endothelial cells (arrows) in a representative liver section are shown (a, ×100; b, ×200; c, ×400), and no positive staining in the livers of mice receiving a single injection of LPS (d, ×200).
2.3. LPS and GSR-induced mice

LPS was extracted from *Klebsiella pneumoniae* O3 LEN-1 by the phenol–water method [14,15]. GSR was induced in mice by two consecutive injections of lipopolysaccharide. The optimal doses of LPS in the preparative and provocative injections were determined by preliminary experiments with reference to previous publications [2–5]. The optimal dose of LPS (5 μg) was injected intradermally into the footpads of mice as a preparative injection for priming of GSR. 24 h later, a provocative injection of LPS (300 μg) was administered intravenously. More than 80% of those mice were dead within 12 h after the provocative injection of LPS. Three to four mice of BALB/c strain were used in each experimental group.

2.4. In situ specific labeling of fragmented DNA

Mice were killed 7 h after challenge with LPS unless otherwise stated, and various organs were collected. The liver, kidney and lung were primarily studied because they are LPS-susceptible organs. The tissues were fixed with formalin, and cut serially to 4–6 μm sections. The sections were deparaffinized for in situ nick end labeling specific for fragmented DNA. The technique reported originally by Gavrieli et al. [16] was used as described previously [10]. Nick end labeling defines apoptotic cells carrying fragmented DNA [16]. Vascular endothelial cells were mainly identified by their morphological characteristics, and in some cases they were stained with anti-vascular cell adhesion molecule-1 antibody.

3. Results

3.1. Induction of apoptosis of vascular endothelial cells in GSR-induced mice

LPS (5 μg) was injected into footpads of 4 mice as a preparative injection, and 24 h later LPS (300 μg) was injected intravenously as a provocative injection. Sections of the liver, lung, and kidney 7 h after the provocative injection were stained by nick end labeling specific for fragmented DNA. Typical experimental results are shown in Fig. 1 and 2.

![Fig. 1. Vascular lesions in the liver of GSR-induced mice. The liver sections of GSR-induced BALB/c mice were stained with hematoxylin and eosin (a) or nick end labeling (b). Fragmented nuclei (a) and positively stained nuclei (b) of vascular endothelial cells are indicated by arrows. X 1000.](https://academic.oup.com/femspd/article-abstract/16/3-4/205/515366)

![Fig. 2. Vascular lesions in the liver of GSR-induced mice. The liver sections of GSR-induced BALB/c mice were stained with hematoxylin and eosin (a) or nick end labeling (b). Fragmented nuclei (a) and positively stained nuclei (b) of vascular endothelial cells are indicated by arrows. X 1000.](https://academic.oup.com/femspd/article-abstract/16/3-4/205/515366)

Nuclei of vascular endothelial cells in the livers of all mice were stained positively 7 h after the provocative injection (Fig. 1a,b). There were several positive nuclei in the parenchymal region of the liver. Positively stained endothelial cells were sometimes accompanied by aggregation of leukocytes, platelets and erythrocytes, and the leukocytes were also stained positively (Fig. 1c). There were few positively stained nuclei of endothelial cells in the livers of mice receiving a single injection of the provocative dose of LPS (300 μg) (Fig. 1d). Positive cells were hardly detected in the livers of mice primed with 5 μg of LPS and normal control mice (data not shown). In the liver sections stained with hematoxylin and eosin, few visible changes were detected in the morphology of vascular endothelial cells. However, fragmented nuclei were also detected...
at the place in which the nuclei were stained positively by nick end labeling (Fig. 2). The renal tubules in GSR-induced mice were also stained positively (Fig. 3), and the staining pattern of renal tubules was focal at multiple points. In the lung, vascular endothelial cells were positive for nick end staining (data not shown). In addition, vascular endothelial cells in the liver 3 h after the provocative injection were not stained positively, and their positive staining was detectable at 6–7 h after the provocative injection. We tried to detect fragmented DNA with agarose gel electrophoresis. However, we found hardly any, probably because of a small number of positively stained cells in the organs.

3.2. Participation of IFN-γ in priming of vascular endothelial cells for apoptosis

The participation of various cytokines in priming vascular endothelial cells for apoptosis was examined (Fig. 4). In the preparative injection, various anti-cytokine neutralizing antibodies were injected intravenously into mice together with LPS. The induction of apoptosis in endothelial cells was studied 7 h after the provocative injection. Simultaneous administration of anti-IFN-γ antibody (100 μg) with LPS completely inhibited the induction of apoptosis of vascular endothelial cells in all 4 mice, suggesting the participation of IFN-γ in priming for apoptosis (Fig. 4b). On the other hand, anti-TNF-α antibody (100 μg) did not significantly inhibit it. Moreover,
administration of anti-IL-2 or IL-1β antibody (100 μg) could not inhibit it, either. Based on the above experiment, the possibility was raised that IFN-γ might play a key role in apoptosis induction of endothelial cells. Therefore, it was examined whether sensitization with IFN-γ instead of LPS caused apoptosis of endothelial cells. Recombinant IFN-γ (2 μg) was injected into footpads of mice for sensitization, and 24 h later a provocative injection of LPS was given intravenously to those mice. The induction of apoptosis was studied by nick end labeling. Priming with recombinant IFN-γ definitely caused apoptosis of vascular endothelial cells (Fig. 4c). However, its degree was less compared to that of LPS.

3.3. Effector molecules in apoptosis of vascular endothelial cells by the provocative injection of LPS

We studied whether cytokines participated as effector molecules in induction of apoptosis of vascular endothelial cells by the provocative injection of LPS. Mice were primed with a preparative injection of LPS, and 24 h later they were challenged with a provocative injection of LPS together with various anti-cytokine neutralizing antibodies. Administration of anti-TNF-α antibody (100 μg) together with LPS partly reduced the number of positively stained vascular endothelial cells. On the other hand, administration of anti-IL-2, IL-1β or IFN-γ antibody alone did not affect it. To confirm the role of TNF-α on apoptosis induction, a challenge with recombinant TNF-α (2.5 μg) was given intravenously to LPS-primed mice. However, administration of recombinant TNF-α instead of LPS did not induce apoptosis of vascular endothelial cells (data not shown). On the other hand, fragmented nuclei of renal tubules in those mice were stained positively by nick end labeling (Fig. 5). There were much more apoptotic cells of renal tubules in mice injected with recombinant TNF-α than in mice injected with LPS. Positive staining of renal tubules was not seen when recombinant IL-1β was administered. Although various anti-nitric oxide or superoxide agents were tried for inhibition of apoptosis of vascular endothelial cells, none of them significantly inhibited apoptosis.

4. Discussion

In this study, we have demonstrated that the nuclei of vascular endothelial cells in GSR-induced mice which were produced by two consecutive injections of LPS were stained positively by in situ specific labeling of fragmented DNA. Moreover, fragmented nuclei of vascular endothelial cells were detected. These findings suggested that systemic cell death of vascular endothelial cells in GSR was caused by apoptotic cell death. However, we could not exclude the participation of necrotic cell death in the present study because it is very difficult to discriminate exactly between necrosis and apoptosis. The apoptotic cell death of vascular endothelial cells
could not be produced by a single injection of LPS. Further, it was undetectable when LPS was administered to normal mice or D-galactosamine-sensitized mice [9–11,13]. Therefore, the apoptosis of vascular endothelial cells might be closely related to GSR, which is characterized by vascular occlusion, hemorrhage, and perivascular accumulation of leukocytes, followed by injury and necrosis of endothelial cells [1,2,5]. Since GSR is known as an experimental DIC model [5], systemic damage of vascular endothelial cells in clinical DIC might also be caused by apoptosis.

Acute tubular necrosis is a typical sign of endotoxic shock and DIC [17]. However, it was found that cells undergoing apoptosis were detected focally in renal tubules of GSR-induced mice. This focal apoptosis of renal tubules might be referred to as acute tubular necrosis in endotoxic shock and multiorgan failure [9]. Further, administration of recombinant TNF-α instead of LPS resulted in more marked apoptosis of renal tubular cells. It is possible that TNF-α plays an important role in the induction of acute tubular necrosis in endotoxic shock and DIC. The participation of TNF-α in damage of renal tubules suggested that acute tubular necrosis might be caused by apoptosis, because TNF-mediated cell death is known to be apoptotic [6–8].

Simultaneous administration of anti-IFN-γ antibody in the preparative injection of LPS completely prevented the apoptosis of vascular endothelial cells. However, administration of the antibody in the provocative injection did not affect it. Therefore, it was suggested that IFN-γ might play a key role in priming vascular endothelial cells for apoptosis. This idea was confirmed by the fact that administration of recombinant IFN-γ as the preparative injection could reproduce the apoptosis of endothelial cells. IFN-γ should be a key molecule for sensitization of endothelial cells for apoptosis. This is consistent with several reports stating that IFN-γ plays a crucial role in the lethal activity of LPS against GSR-induced mice [3,18–20]. Based on the fact that IFN-γ sensitizes mice to the lethality of GSR and the apoptosis of vascular endothelial cells, and that anti-IFN-γ antibody prevents both, the apoptosis of vascular endothelial cells might be involved in the lethality of GSR. The role of IFN in priming vascular endothelial cells for apoptosis should be clarified.

What is the terminal molecule for apoptosis induction of endothelial cells in GSR-induced mice? There are several reports on the terminal molecules in challenge of LPS for the lethality of GSR in mice [18,19,21]. TNF-α, IL-1β and IFN-γ have been reported to be involved in the lethality of GSR [18,19,21]. However, the injection of recombinant TNF-α, IL-1β or IFN-γ and their combination did not cause apoptosis of endothelial cells. This finding is not necessarily consistent with the role of cytokines in the lethality of GSR [21]. Probably, a number of LPS-induced noxious molecules are involved in apoptosis induction of endothelial cells in GSR. This indicates that the apoptosis of vascular endothelial cells might require LPS in the provocative injection, and that LPS could not be entirely replaced by cytokines.

The in situ specific labeling of fragmented DNA was applied to characterize the injuries of vascular endothelial cells in GSR. This method clearly stained the nuclei of vascular endothelial cells in GSR-induced mice positively even when there were few visible changes in classically hematoxylin and eosin-stained sections. Therefore, this staining method might be useful for detecting the earliest morphological damages of vascular endothelial cells in GSR and DIC.

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References


