Induction of apoptotic cell death by pancreatitis-associated ascitic fluid in Madin–Darby canine kidney cells

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Abstract **We investigated the cytotoxicity on Madin–Darby canine kidney (MDCK) cells of pancreatitis-associated ascitic fluid (PAAF) collected from rats with experimental necrotizing pancreatitis. PAAF reduced viability of MDCK cells in a time- and dose-dependent manner. We detected DNA fragmentation on the PAAF-treated MDCK cells, indicating that the cytocidal action of PAAF is via apoptosis. From the results obtained, we conclude that PAAF contains factor(s) inducing apoptosis on MDCK cells, and we assume that apoptotic cell death is involved in the mechanism of organ failure in acute pancreatitis.**

Key words: Acute pancreatitis; Ascitic fluid; Apoptosis; Multiple organ failure; Madin–Darby canine kidney cell

1. Introduction

In patients with acute pancreatitis, especially the severe form, the interaction of the impact on the human body and the response in terms of defence mechanisms result in a number of consequences. Among these, multiple organ failure (MOF) is the most serious complication in the early stage of this disease, and numerous efforts have been made to clarify the mechanism of organ failure in acute pancreatitis.

From the 1960s, ascitic fluid has been noticed to play an important role on clinical progression of acute pancreatitis [1–4], and Pickford et al. [5] initially reported a relationship between findings on abdominal paracentesis and peritoneal lavage at the time of diagnosis, and the prognosis of human acute pancreatitis. Other than diagnostic values, many investigators followed Ranson's report [6] showing the therapeutic efficacy of peritoneal lavage. As for the mechanism of its involvement, a number of experimental reports have been made as follows. Frey et al. [7] showed that the pancreatitis-associated ascitic fluid (PAAF) from the canine model of necrotizing pancreatitis had a lethal effect on the rats when administered into their abdominal cavity. Coticchia [8] demonstrated that clinical PAAF blocked the respiration of the liver mitochondria isolated from rats in vitro. Bielecki [9] showed the same effect of the PAAFs in both canine models and human cases. Satake [10] reported that PAAF from a canine pancreatitis model caused acute renal failure suggesting that the nephrotoxic substance existed in PAAF.

On the other hand, apoptosis was initially recognized as a type of cell death that serves to eliminate excessive or unwanted cells [11–13]. Recently, it has been reported that apoptosis is induced not only in the course of organ development but also in cellular injury such as inflammatory disease or administration of anti-tumor chemotherapeutic drugs [14–17]. It has also been reported that cytokines, such as interleukin-1 (IL-1) [18], tumor necrosis factor α (TNF-α) [19], transforming growth factor-β (TGF-β) [20] and nitric oxide [21], can induce apoptosis. As has been clarified that various cytokines including IL-6, IL-8 and TNF-α are released into peritoneal exudate and peripheral circulation, it is highly possible that apoptotic cell death is involved in the mechanism of MOF in acute pancreatitis. Along the line of these observations, we have hypothesized that PAAF induces apoptotic cell death.

In the present study Madin–Darby canine kidney (MDCK) cells were selected as target cells to test the hypothesis. The MDCK cell is the established cell line of normal distal tubular cells from canine renal parenchyma, and it has been already reported that apoptotic cell death is inducible on this cell line by hypoxia [22], infection of influenza viruses [23], or the treatment with TGF-β [24] or toxins such as ricin.

We will demonstrate that PAAF contained the substance(s) inducing cell death via apoptosis, using MDCK cells as target cells, and discuss the possible mechanism of inducing apoptosis by PAAF.

2. Materials and methods

2.1. Materials and chemicals

Male Wistar rats (250 g wt) were employed for the preparation of PAAF. MDCK cells were obtained from the Japanese Cancer Research Resources Bank. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco-BRL (MD, USA). Fetal calf serum (FCS) was from CSL (Australia). Streptomycin sulfate and Penicillin G were from Meiji (Tokyo, Japan) and from Banyu (Tokyo, Japan), respectively. Sodium deoxycholate (DCA) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 3-(4,5-dimethylthiazole 2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular proliferation assay kit was purchased from Chemicon, Inc. (CA, USA). Flat-bottomed plates with 96 wells were from Corning (NY, USA). DNase-free RNase and the cellular DNA fragmentation ELISA kit were from Boehringer Mannheim (Germany). Other materials and chemicals were obtained from commercial sources.

2.2. Cell culture

MDCK cells were cultured in DMEM with 10% FCS and antibiotics, streptomycin sulfate (100 μg/ml) and penicillin G (100 U/ml) in a water-saturated atmosphere of 5% CO_2 in air at 37°C.

2.3. Preparation of PAAF

Experimental necrotizing hemorrhagic pancreatitis was induced in 10 male Wistar rats under general anesthesia by the retrograde injection of 0.1 ml of 30% DCA in water into the pancreatic ducts. The peritoneal exudate was aseptically collected from the abdominal cavities 5 h later,

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and was centrifuged at 2,500 × g for 15 min at 4°C and then the supernatant was collected together from 10 rats as PAAF. The average volume of the PAAF from one animal was approximately 8 ml. Endotoxin was not detected in the PAAF by endospacy and toxicolor methods.

2.4. Cellular viability

The cytotoxic effects of the PAAF on MDCK cells were quantified using an MTT cellular proliferation assay as described previously [25]. In brief, MDCK cells were seeded at 1×10^4 cells/well (0.1 ml) in a flat-bottomed plate with 96 wells in DMEM with 10% FCS and, attached to the plates. Then the cells were cultured with various concentrations of the PAAF (0, 10, 20 and 30%) in DMEM without FCS and incubated for various periods of time (4–24 h) at 37°C. Then another 4 h incubation with 0.01 ml of MTT reagent at 37°C followed. After the incubation, the incubation medium was discarded, and the precipitates were dissolved in 0.1 ml of DMSO. The absorbance was measured spectrophotometrically at 570 nm using a microplate reader.

2.5. DNA extraction and gel electrophoresis

MDCK cells were seeded on 60 mm dish and cultured to confluence, then the cells were incubated with DMEM containing 20% PAAF without FCS for various periods of time. The cells were lysed with 0.1 M NaCl, 10 mM Tris HCl (pH 8.0), 5 mM EDTA in 0.5% SDS, and incubated overnight at 52°C with proteinase K (100 mg/ml). The samples were extracted with an equal volume of phenol/chloroform (1 : 1) and the total DNA in the aqueous phase was precipitated with 1/10 volume of 100% ethanol at −20°C overnight. The DNA pellets were obtained by centrifugation (12,000 × g, 15 min), and washed with 70% ethanol, air dried, and resuspended in 0.1 ml of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. The samples were treated with DNase-free RNase (10 units/ml) for 30 min at 37°C, and applied to electrophoresis using a 1.5% agarose gel containing ethidium bromide.

2.6. Enzyme-linked immunosorbent assay (ELISA) of cellular DNA

Cellular DNA fragmentation was quantified using a cellular DNA fragmentation ELISA kit as described elsewhere [26] with slight modifications. In brief, MDCK cells were prelabeled with 1 mM 5-bromo-2′-deoxyuridine (BrdU) in DMEM overnight. After labeling with BrdU, the cells were transferred to a flat-bottomed plate with 96 wells at a density of 1×10^4 cells/well, and incubated in DMEM without FCS with various concentrations of the PAAF (0, 10, 20 and 30%) at 37°C. After various periods of time, the cells were lysed by adding lysis buffer. The cellular DNA fragments in the lysates were collected by the method as described [26], and quantified by measuring photometrical peroxidase activity associated with BrdU, which was labeled to the DNA fragments (the absorbance at 450 nm, reference wavelength: 690 nm).

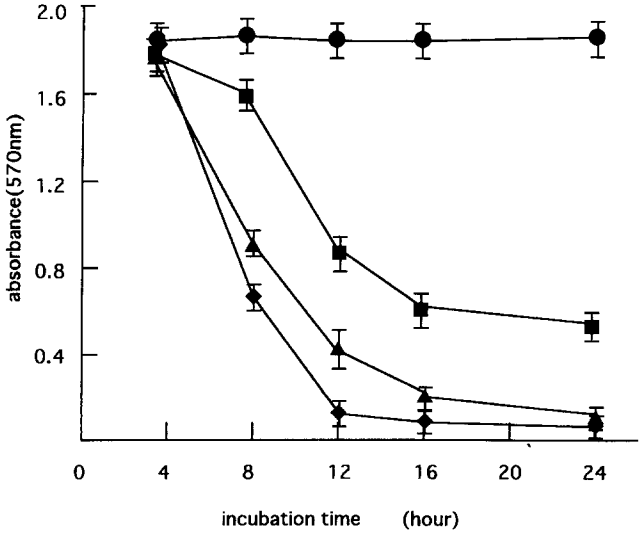

Fig. 1. The effects of PAAF on the viability of MDCK cells. Cell viability was assayed using MTT assay kits as described in section 2. The cells were incubated in DMEM with or without the PAAF for 4–24 h. (●), without the PAAF; (■), with 10% PAAF; (▲), with 20% PAAF; (◆), with 30% PAAF. The values represent mean ± S.D. of results of eight independent experiments.

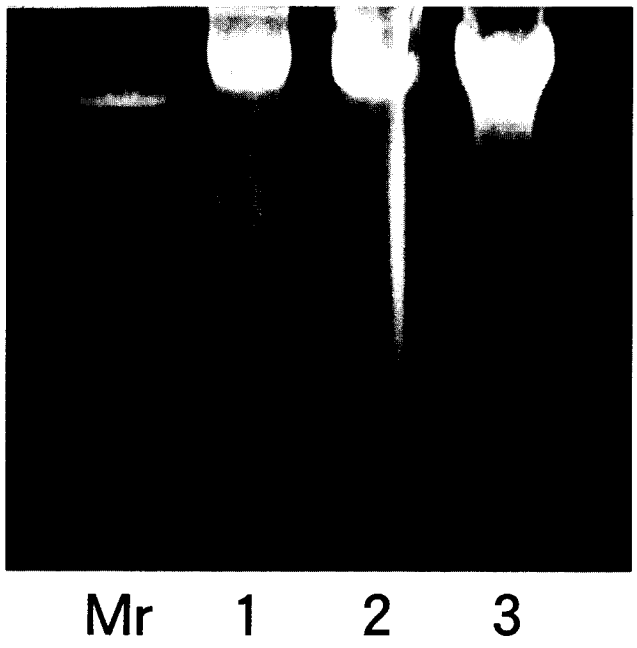

Fig. 2. Induction of DNA fragmentation by PAAF. Cellular DNA was collected from MDCK cells incubated with or without PAAF. The collected DNA was electrophoresed on a 1.5% agarose gel containing 0.5% ethidium bromide. Lane 1, incubated with 20% PAAF for 8 h; lane 2, with 20% PAAF for 12 h; lane 3, without the PAAF for 12 h; Mr, an *Eco*RI–*Hin*dIII digest of λ phage DNA loaded as a molecular weights standard.

3. Results

3.1. MTT assay

The cytotoxic effects of PAAF on MDCK cells were evaluated using an MTT assay (Fig. 1). MDCK cells were exposed to PAAF (0, 10, 20, 30%) in DMEM without FCS culture medium, and incubated for various periods of time. Although the viability of the MDCK cells was not altered without PAAF along the period of incubation, the decrease in viability was noticed after 8 h of incubation with all concentrations of PAAF and progressed along with incubation. The viability of the cells was almost 0% after 24 h incubation with 20% and 30% PAAF, and a dose-dependent effect of PAAF was observed during the incubation.

3.2. DNA fragmentation on agarose gel electrophoresis

Fig. 2 shows the electrophoretic patterns of cellular DNA of the MDCK cells after treatment with and without PAAF for 8 and 12 h. DNA fragmentation became apparent as a step-ladder pattern of the cellular DNA from the cells treated with 20% PAAF for 8 and 12 h (lane 1 and 2), but no step-ladder pattern was detected on the cellular DNA from the cells without PAAF treatment (lane 3).

3.3. Cellular DNA ELISA

MDCK cells were incubated with various concentration of the PAAF (0, 10, 20 and 30%) for 4–18 h. Without the PAAF

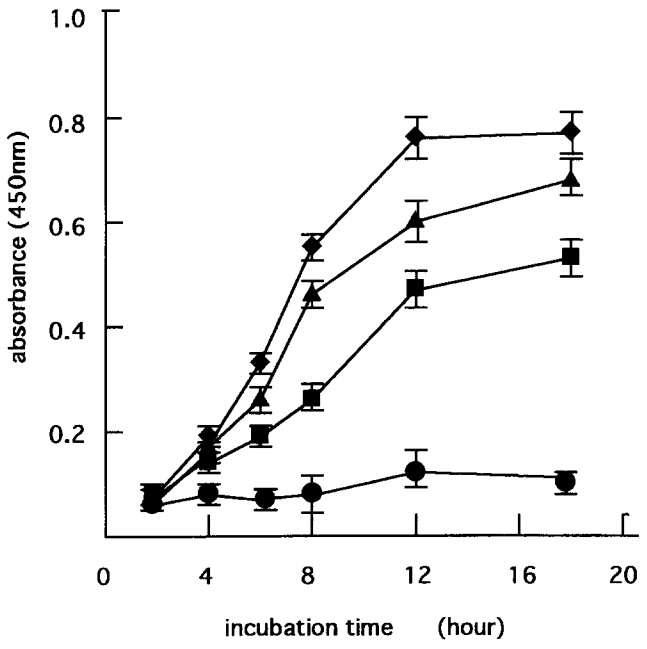

Fig. 3. Increase of DNA fragmentation of MDCK cells by PAAF. DNA fragmentation was quantified using a cellular DNA fragmentation ELISA kit as described in section 2. The cells were incubated in DMEM with or without PAAF for 2–18 h. (●), without the PAAF; (■), with 10% PAAF; (▲), with 20% PAAF; (◆), with 30% PAAF. The values represent the mean ± S.D. of results of four independent experiments.

treatment (control), the DNA fragments did not increase during the time of incubation, but with PAAF, the DNA fragments began to increase after 4 h (Fig. 3). The DNA fragments increased in a linear manner until 12 h and reached maximal levels with PAAF (Fig. 3). The DNA fragments were induced by the addition of PAAF in MDCK cells in a dose- and time-dependent manner.

4. Discussion

Apoptotic dying cells exhibit characteristic changes, condensation of nuclear chromatin in the nuclear periphery, cell membrane blebbing, the formation of apoptotic bodies, and internucleosomal cleavage of DNA [27–29]. Internucleosomal fragmentation of chromatin DNA has universally been accepted as a criterion for the definition of apoptotic cell death [30]. As shown on the agarose gel electrophoresis in the present study, the DNA extracted from PAAF-treated MDCK cells was electrophoresed in a step-ladder pattern, indicating the existence of DNA fragmentation. In addition, the cellular DNA ELISA detected DNA fragments in a dose- and time-dependent manner. At the same time, the MTT assay showed the cytocidal effect of PAAF on MDCK cells, and the similarity the dose- and time-dependent manner in the cellular DNA ELISA and those in the MTT assay suggests that the cytocidal effect of PAAF observed in the present study is derived from apoptotic cell death.

There are two possible mechanisms inducing apoptosis in the system presented here. One is a non-specific mechanism such as hypoxic stress as reported previously on MDCK cells [22]. As the existence of interfered substance(s) against mitochondrial respiration in PAAF has been documented by several researchers [8,31,32], the possibility cannot be neglected that apoptosis is induced by putative substance(s) interfering mitochondrial respiration. However, since it was reported that it took 24 to 48 h to induce apoptosis by hypoxic stress on MDCK cells, the apoptosis by PAAF observed here is completed too fast in order to be judged as induced by non-specific stimulations such as hypoxic stress. Another possible mechanism is that the signal for apoptosis is transferred by specific factor(s) in PAAF such as cytokine(s) to the cell membrane and induces apoptosis by the activation of specific intracellular signal transduction system(s). For instance, TGF-β, one of the cytokines released from various cells or tissues including activated macrophages, was reported to induce apoptosis on MDCK cells [24]. On the other hand, the cytokines and their derivatives, which were released from the activated macrophages or lymphocytes, and elastase released by polymorphonuclear leukocytes play important roles in the induction of organ failure in acute pancreatitis [33–35]. Considering these lines of evidence, it is highly possible that the apoptosis-inducing activity in PAAF on MDCK cells is via cytokine(s) or their derivatives.

As far as we can survey, there are no reports stating the relation between apoptosis and MOF, even if we not limit ourselves to acute pancreatitis. However, apoptosis has occurred not only in a normal physiological process but also under pathological conditions, such as administration of antitumor agents [36,37], viral infections [38,39], or autoimmune disease [40–43]. Here, we have described for the first time the possible involvement of apoptotic cell death in the mechanism of MOF, particularly in acute pancreatitis. In order to identify the factor(s) inducing apoptosis in acute pancreatitis, further investigations will have to be performed not only in vitro but also in in vivo experiments.

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