In Vitro Study of Neutrophil Apoptosis in Liver Cirrhosis

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Cirrhotic patients frequently manifest neutropenia and are predisposed to bacterial infections. We examined neutrophil apoptosis to determine if neutrophil survival in cirrhotic patients is shortened. Neutrophils isolated from 10 cirrhotic patients and 10 healthy volunteers were cultured for 24 hours. The time course of neutrophil viability was assessed by the trypan blue dye exclusion test and apoptosis was determined morphologically by light and electron microscopy. Apoptotic cells were also confirmed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick and labeling (TUNEL) and DNA gel electrophoresis. Fas expression of neutrophils was examined by flow cytometry. Viabilities were significantly decreased in liver cirrhosis ($p<0.0001$). Neutrophils from cirrhotic patients exhibited significantly greater apoptosis. Fas expression of neutrophils was significantly reduced for cirrhotic patients ($p=0.0001$). Neutrophils from cirrhotic patients exhibited markedly accelerated apoptosis *in vitro*. Shortening of neutrophil survival via apoptosis may explain in part the mechanism of neutropenia in cirrhotic patients. (Internal Medicine 37: 11–17, 1998)

Key words: cell death, leukopenia, Fas, DNA analysis, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick and labeling (TUNEL)

Introduction

Cirrhotic patients frequently exhibit anemia, leukopenia, and thrombocytopenia. Several mechanisms underlying anemia such as folate deficiency, hypersplenism and hemolysis, such as in spur cell anemia, have been described (1–3). Hypersplenism and shorter life span of thrombocytes have been shown to cause thrombocytopenia (2, 4, 5). In the pathogenesis of neutropenia, splenomegaly and serum hematopoietic progenitor cell inhibitory factors have been reported (6–9). However, an accurate kinetic study of neutrophils is difficult to perform because the half-life of a neutrophil may be less than 7 hours (10) in healthy subjects.

Recently, apoptosis has been acknowledged as a physiological suicide mechanism and many cell types undergo programmed death via apoptosis to maintain homeostasis (11). The apoptosis of neutrophils *in vitro* was first reported by Savill et al (12). Grigg et al observed many apoptotic neutrophils phagocytosed by alveolar macrophages (13). The liver and the spleen were also shown to be important organs for the disposal of apoptotic neutrophils, as reported by Shi et al (14). We assumed that accelerated neutrophil apoptosis led to shortening of neutrophil survival and may be partly involved in neutropenia in cirrhotic patients. To clarify the issue described above, we

cultured neutrophils and compared the frequency of neutrophil apoptosis in cirrhotic patients and controls.

Methods

Subjects

Healthy volunteers ($n=10$) and cirrhotic patients with a neutrophil count of less than $2,000/\text{mm}^3$ ($n=10$) were enrolled. The diagnosis of liver cirrhosis was based on clinical and laboratory findings, including liver biopsy. Patients with cirrhosis complicated by hepatocellular carcinoma of less than 2 cm in diameter were included. Patients with fever, positive C-reactive protein, or who were under treatment were excluded. The cirrhotic patient group included 3 men and 7 women (42–75 years of age). Control subjects included 6 men and 4 women (35–80 years of age). The cause of liver cirrhosis was hepatitis C virus in 8 patients and hepatitis B virus in 2 patients. Informed consent was obtained from every patient and the study was approved by the ethics committee of Kurume University.

Neutrophil isolation and culture

Heparinized blood samples from a peripheral vein were mixed with an equal volume of phosphate-buffered saline (PBS, pH 7.4). Leukocytes were separated from erythrocytes

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by dextran sedimentation using 0.6% dextran (Wako Pure Chemical Industries, Osaka) for 30 minutes. The leukocyte-rich upper layer was fractionated using a modification of the discontinuous plasma-percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient technique described by Haslett et al (15). After centrifugation at 200g for 30 minutes at room temperature, neutrophils were collected at the interface, washed twice in PBS, and resuspended at a concentration of $5.0 \times 10^6/\text{ml}$ in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo). The medium was supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, N.Y.), 0.2 mM L-glutamine (Nissui), 25 U/ml penicillin (Meiji, Tokyo), and 25 mg/ml streptomycin (Meiji). The neutrophils were $\geq 95\%$ pure by May-Giemsa staining and $\geq 98\%$ viable by trypan blue dye exclusion. The cell suspensions were seeded into 6-well flat-bottom plates (Falcon 3046, Becton Dickinson and Company, Lincoln Park, New Jersey) and cultured at 37°C in $5\% \text{CO}_2$ for 24 hours.

Neutrophil viability

At each designated time, neutrophil viability was assessed by trypan blue dye exclusion. One vol of 0.4% trypan blue was added to 5 vol of cell suspension. After incubation at room temperature for 5 minutes, cells were counted in a hemocytometer. All counts were performed in triplicate with coded samples.

Light microscopy

Cytospins were prepared using a Shandon Cytospin 2 (Shandon Inc., Pittsburgh, PA) and stained with May-Giemsa stain. A total of 500 cells were counted by light microscopy at a high magnification (1,000 $\times$). Apoptotic cells were identified according to the following criteria of Savill et al (12): condensed or fragmented nuclei, cytoplasmic vacuolation, and decrease in cell size.

Transmission electron microscopy

Neutrophils cultured after 24 hours were fixed in 1% glutaraldehyde in 0.05 M cacodylate buffer and 7.5% sucrose in 0.05 M cacodylate buffer for 3 hours. The samples were postfixed with 1% osmium tetroxide for 1 hour, dehydrated, and embedded in epoxy resin (Nissin EM, Tokyo). Thin sections were cut, double contrast-labeled with uranyl acetate and lead citrate, and examined with an electron microscope (H-7,000, Hitachi) at 75 kV.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick and labeling (TUNEL) assay

The TUNEL assay (16) was employed to observe DNA fragmentation in neutrophils. After 12 hours of culture, neutrophils were collected on poly-L-lysine-coated glass slides (Sigma, Chemical Co., St Louis, MO) using cytospin. The samples were fixed with 4% fresh formaldehyde solution in PBS. After washing twice with PBS, the samples were permeabilized in Triton X (Wako) solution in PBS. After being washed with PBS, the fragmented DNA of neutrophils were

labeled with fluorescein-12-dUTP using an Apoptosis Detection System, Fluorescein (Promega Corporation, Madison, WI) according to the manufacturer's protocol. Subsequently, the samples were counterstained with propidium iodide (1 $\mu\text{g}/\text{ml}$; Sigma), and analyzed by confocal laser microscopy (Olympus LSM-GB 200, Tokyo).

DNA electrophoresis

Neutrophils (5.0×10^6 cells) were removed from culture after 12 hours and 24 hours, collected into Eppendorf tubes, and washed twice with cold PBS. The samples were resuspended in 400 μl DNA extraction buffer (10 mM Tris HCl PH 8.0, 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 2% SDS). Proteinase K (500 $\mu\text{g}/\text{ml}$; Boehringer Mannheim GmbH, Mannheim, Germany) and RNase (20 $\mu\text{g}/\text{ml}$; Sigma) were added, and these suspensions were incubated for 18 hours at 37°C . DNA was extracted twice by means of phenol-chloroform, then precipitated with 0.1 vol of 3 M sodium acetate (Nippon Gene Co., Tokyo) and 2.5 vol of 100% cold ethanol, and stored for 1 hour at -70°C . The precipitates were rinsed with 70% ethanol and dried. DNA-pellets were resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA PH 8.0) and heated for 10 minutes at 70°C . DNA (10 μg) was applied to each lane on 1% agarose gel containing 1.0 $\mu\text{g}/\text{ml}$ ethidium bromide (Nippon Gene). The electrophoresis was performed for 220 minutes at 50 V, and the gel was photographed under UV light. The size of DNA fragments in the samples was compared with a standard ladder (GIBCO).

Expression of Fas antigen

All incubations were performed using PBS containing 1% bovine serum albumin (Wako) and 0.1% sodium azide (binding buffer). Fresh isolated neutrophils were washed at 4°C in binding buffer and suspended (5×10^5) in 50 μl binding buffer. The cells were incubated with FITC-conjugated anti-Fas monoclonal antibody (clone UB-2; MBL, Nagoya) or an isotype-matched FITC-conjugated mouse IgG1 mAb (Becton Dickinson, Mountain View, CA) for 15 minutes at room temperature. The cells were washed twice with binding buffer at 4°C and fixed with 1% paraformaldehyde in PBS. The stained cells were immediately analyzed on a Cytron Absolute flow cytometer (Ortho Diagnostic systems, Tokyo).

Statistical analysis

Results are expressed as the mean $\pm$ standard deviation. To assess survival and apoptosis rates, the repeated measure ANOVA was used. Fas antigen expression data were analyzed by Mann-Whitney's U test. Statistical significance was defined as $p < 0.05$.

Results

Neutrophil viability in control and cirrhotic patients

After percoll gradient centrifugation, neutrophil viabilities in both control and cirrhotic patients exceeded 98%. Neutrophil viabilities in cirrhosis and controls were 96.3 ± 2.2 and $98.5 \pm$

1.4% at 4 hours, 91.4 ± 5.4 and $97.1 \pm 1.8\%$ at 8 hours, 79.6 ± 5.8 and $89.3 \pm 4.5\%$ at 12 hours, and 34.5 ± 13.1 and $53.5 \pm 12.6\%$ at 24 hours, respectively (Fig. 1). There was a significant difference ($p < 0.0001$) in viability between cirrhotic patients and control subjects.

The light microscopic evaluation of apoptosis

Neutrophils exhibiting the morphological changes of apoptosis are demonstrated in Fig. 2A and B. In fresh isolated neutrophils from cirrhotic patients, 1.8% of neutrophils had

Figure 1. Time course for the viability of cultured neutrophils in control subjects and in liver cirrhosis. Cell viability was assessed by the trypan blue dye exclusion test. The results are expressed as mean $\pm$ SD. Data were analyzed by repeated measure ANOVA. Neutrophil viabilities were significantly ($*p < 0.0001$) decreased for cirrhotic patients ($n=10$) compared to control subjects ($n=10$).

Figure 2. Morphological features of neutrophil apoptosis during culture. Light microscopic examination of neutrophils cultured for 8 hours ($\times 1,000$). (A) Control neutrophils, and (B) neutrophils from a cirrhotic patient. Cells with highly condensed or fragmented nuclei and vacuolated cytoplasm were considered apoptotic. The number of apoptotic neutrophils was markedly greater in liver cirrhosis than in controls. (C) Time course for apoptosis of cultured neutrophils in control and liver cirrhosis. The results are expressed as mean $\pm$ SD. Data were analyzed by repeated measure ANOVA. Apoptosis was significantly ($*p < 0.05$) increased for neutrophils from cirrhotic patients ($n=10$) compared to control neutrophils ($n=10$).

already exhibited morphological features of apoptosis (data not shown). The incidences of apoptotic neutrophils in cirrhotic patients and controls were 4.6 ± 2.9 and $1.5 \pm 1.4\%$ at 4 hours, 40.9 ± 20.6 and $17.3 \pm 14.5\%$ at 8 hours, 59.3 ± 23.1 and $37.4 \pm 16.8\%$ at 12 hours, and 24 hours 92.4 ± 6.9 and $78.9 \pm 15.8\%$ at 24 hours, respectively (Fig. 2C). Statistical significance for control and cirrhotic patients was demonstrated at every time point in culture ($p < 0.05$).

Electron microscopic evaluation of apoptosis

More than 90% of cirrhotic neutrophils cultured for 24 hours demonstrated condensed chromatin or formed crescents of chromatin, rounded nuclear profiles, cytoplasmic vacuolations, increase in the electron density of the cytoplasm, cell shrinkage, and loss of microvilli and pseudopodia. However, the plasma membrane and cellular organellas remained intact (Fig. 3). These cells are characteristic of apoptotic cells (17, 18).

TUNEL assay

Nuclei with fragmented DNA were labeled green with fluorescein-12-dUTP and the cells were counterstained with propidium iodide in red. TUNEL-positive cells appeared as intensively yellow fluorescent cells at confocal laser microscopy. A much larger population of neutrophils of cirrhotic patients were TUNEL-positive as compared to controls (Fig. 4). TUNEL-positive cells with intense fluorescence had round nuclei with no lobules.

Electrophoretic analysis of DNA

Figure 5 shows representative results of ten separate experiments. In cirrhotic patients, a ladder of DNA fragments of 180–200 bp was clearly exhibited. The ladder formation was more clearly observed in neutrophils from cirrhotic patients than in those from controls during the entire experimental period up to 24 hours.

Expression of Fas antigen on neutrophils

Expression of Fas antigen on neutrophils freshly isolated from control and cirrhotic patients was evaluated by flow cytometric analysis. Neutrophils from cirrhotic patients expressed significantly less Fas antigen than did those from controls (39.6 ± 13.4 vs. $75.7 \pm 17.6\%$, $p = 0.0001$). Figure 6B demonstrates the representative histograms. The histograms illustrate that neutrophils from controls have a markedly larger population with a high fluorescence intensity than did neutrophils from cirrhotic patients.

Discussion

The neutrophil has the shortest life span among white blood cells and it is difficult to confirm its kinetics in the body. So, it is not easy to elucidate the mechanism of neutropenia associated with liver cirrhosis. Its elucidation is important because of the predisposition of cirrhotic patients to bacterial infection (19, 20). Hypersplenism has been regarded as a major cause of neutropenia in cirrhosis. However, neutropenia does not always

Figure 3. Electron microscopic observation of neutrophils cultured for 24 hours ($\times 5,000$). A neutrophil shows condensed chromatin and the formation of crescents of chromatin while the cell membrane and organellas remain intact.

Figure 4. TUNEL stain of neutrophils cultured for 12 hours in control and liver cirrhosis. Neutrophils were observed by confocal laser microscopy ($\times 800$). (A) control neutrophils, and (B) neutrophils from cirrhotic patients. The number of TUNEL-positive cells was markedly greater in cirrhotic patients than in controls.

improve with splenic embolization therapy (21) or splenectomy (2, 22). The role of hypersplenism in the neutropenia of cirrhosis remains controversial.

We demonstrated in cirrhotic patients, decreased neutrophil viability and neutrophil changes compatible with apoptosis, using the TUNEL method and assessing the DNA laddering at 180–200bp, a biochemical hallmark of apoptosis (23–28).

These findings suggest that neutrophils from cirrhotic patients have a shorter life span via apoptosis than do control neutrophils.

Mature human neutrophils undergo spontaneous apoptosis more rapidly, compared to hematocytes such as monocytes and eosinophils in vitro (29). Savill et al reported that apoptotic neutrophils are swiftly recognized and phagocytosed by macrophages (12, 30). A recent study on neutrophil kinetics in OK-432-treated rats indicated that apoptotic neutrophils were phagocytosed mainly by Kupffer cells or macrophages in the hepatic sinusoids, showing that the liver serves a major role in the clearance of apoptotic neutrophils (14). If accelerated neutrophil apoptosis can be observed in vivo, it may help explain the neutropenia in human cirrhosis, because the number of apoptotic neutrophils recognized and removed by phagocytes is increased. It has been reported that the neutropenia in patients with chronic splenomegaly may be caused either by increased destruction of granulocytes in the spleen or increased margination within the spleen (8). Hypersplenism may lead to rapid clearance of apoptotic neutrophils and this may partly explain the neutropenia in cirrhotic patients with splenomegaly. However, the neutropenia in cirrhotic patients is due not only to accelerated neutrophil apoptosis but also to other mechanisms. We should investigate granulopoiesis in cirrhotic patients and

Figure 5. Gel electrophoresis of DNA from cultured neutrophils. Total DNA was extracted (see Methods) and applied on a 1% agarose gel containing ethidium bromide. Lane M is a standard 100-base pair DNA ladder. Lanes 1 and 3 contain DNA from control neutrophils at 12 hours and 24 hours, respectively, and lanes 2 and 4 contain DNA from cirrhotic neutrophils at the same time points. The characteristic DNA ladder formation appeared at 12 hours more clearly in cirrhotic patients.

whether or not many neutrophils in cirrhotic patients are distributed in marginal pools.

Several hypotheses regarding the mechanisms for accelerated apoptosis of neutrophils have been advocated. It has been demonstrated recently that the Fas/Fas ligand system may regulate neutrophil apoptosis (29, 31). The Fas antigen has been identified as the putative surface molecule capable of transducing apoptotic signals into cells (32). We speculated that neutrophils from cirrhotic patients may express Fas antigen more strongly than do control neutrophils. However, the expression of Fas antigen on neutrophils was unexpectedly reduced in the cirrhotic group. One reason for this may be that Fas antigen, as well as Fcγ RIII, may have been shed from the surface of neutrophils during the course of apoptosis. Fcγ RIII, a low affinity receptor for immunoglobulin G, is also known to be shed by neutrophils during apoptosis (33, 34). The neutrophils progressively reduced their Fas expression with time during in vitro culture, in correlation with an increase of neutrophils exhibiting morphological apoptotic changes (data not shown). Another explanation for the decreased Fas antigen expression may be the adaptive down-regulation for chronic neutropenia in cirrhosis. Neutrophil apoptosis can be regulated by mechanisms other than the Fas pathway (29). Therefore, the reduced

Figure 6. Fas antigen expression on freshly isolated neutrophils from control and cirrhotic patients. Neutrophils were stained with FITC-conjugated anti-Fas monoclonal antibody (mAb) and FITC-conjugated mouse IgG1 mAb and analyzed by flow cytometry. (A) Percent Fas expression on neutrophils was lower in liver cirrhosis ($n=15$) than in controls ($n=10$). Results represent mean $\pm$ SD; $*p=0.0001$, control ($75.7 \pm 17.6\%$) versus liver cirrhosis ($39.6 \pm 13.4\%$). Mean flow intensity values for control and liver cirrhosis were 120.5 ± 31.7 and 82.8 ± 4.9 , respectively. (B) Representative histograms of flow cytometric analysis of neutrophils. The open curve represents neutrophils stained with control antibody and the solid histograms represent neutrophils stained with anti-Fas. Control neutrophils were comprised of a markedly greater population having high fluorescence intensity than in cirrhotic patients.

expression of Fas antigen on neutrophils from cirrhotic patients may show that neutrophil apoptosis is not merely mediated by Fas/Fas ligand system. Further study is required to clarify the mechanism of neutrophil apoptosis in liver cirrhosis.

In conclusion, the life span of the neutrophil in cirrhotic patients is shortened by accelerated apoptosis in vitro. This finding may help elucidate the neutropenia in cirrhosis and explain the increased rate of bacterial infections seen in this disease. This study may also provide new insight into neutrophil kinetics in cirrhotic patients.

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