The relationship between apoptosis and splenocyte depletion in rats following ethanol treatment

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Abstract  Splenocyte depletion observed in chronic ethanol-treated rats (ETRs) was studied in relation to apoptosis. The rats were fed with ethanol in a Liber–DeCarli liquid diet (36% of total calories as ethanol) for 7 weeks. Spleens of ETRs and control rats were examined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method, immunohistochemistry using anti-rat p53 and RM4 (specific for macrophages) monoclonal antibodies, and transmission electron microscopy (TEM). The splenic white pulp in ETRs decreased in size and showed a moth-eaten appearance because of the severe depletion of splenocytes. Most TUNEL-positive cells aggregated into clusters or nests and were not isolated in the white pulp of ETRs. The site of RM4 immunoreactivity was consistent with that of clusters of TUNEL-positive cells. The p53 immunoreactivity was observed in apoptotic splenocytes that were isolated or phagocytosed by macrophages. TEM study revealed the increase in tingible body macrophages phagocytosing apoptotic splenocytes in their cytoplasm in ETRs. Chronic ethanol intake certainly induces apoptosis in splenic white pulps, and tingible body macrophages act as both sentinels and scavengers of apoptotic splenocytes expressing p53.

Key words  Apoptosis · Ethanol · Spleen · Macrophage · p53

Introduction

A principal role of apoptosis is the efficient removal of stressed, damaged, or excess cells from tissues without the generation of inflammatory or immune responses. Apoptosis is morphologically characterized by nuclear chromatin condensation, cell shrinkage, budding on the cell surface, and apoptotic body formation. It occurs not only in physiological and developmental events but also in response to cytotoxic agents. The p53 protein, a detector of damaged DNA, inhibits the replication and subsequent division of a cell until the damaged DNA is repaired. However, if DNA repair is not feasible, p53 promotes apoptosis. Loss of functional p53 leads to cellular division with incorrect DNA and malignant transformation.

The tingible body macrophage (TBM), a member of the macrophage family, often phagocytoses lymphocytes within the cytoplasm and is located in the follicular zone of the splenic white pulp (WP), which is basically divided into the following three compartments: periarteriolar lymphoid sheath (PALS), follicular zone, and marginal zone. TBMs are involved in the induction, deletion, and clearance of apoptotic cells under normal and pathological conditions associated with deregulated apoptosis. In experimental animals, chronic alcohol intake is associated with marked atrophy and depletion of lymphocytes from the thymus, blood, and peripheral lymphoid organs. This immunosuppressive effect of alcohol is associated with cancer promotion and increased risk to infectious diseases in both humans and animals. However, the effect of chronic alcohol intake on the spleen has not yet been elucidated with regard to its immunosuppressive effect and possible relationship to TBMs and apoptosis.

In this study, the expression of p53 and the appearance of TBMs and apoptotic splenocytes in chronic ethanol-treated rats (ETRs) were investigated by transmission electron microscopy (TEM), immunohistochemistry, and the terminal deoxynucleotidyl transferase (TdT) -mediated dUTP nick end-labeling (TUNEL) method, and compared with those of control rats.

Materials and methods

Animals and experimental design

Twelve Wistar rats with an approximate average weight of 300g were purchased from SLC-Japan (Shizuoka, Japan).
They were housed under controlled temperature (24° ± 1°C), humidity (55% ± 5%) and lighting (lights on, 0600–1800) conditions. The rats were divided into the following two groups with six rats in each group: one was fed with ethanol in a Liber–DeCarli liquid diet (36% of total calories as ethanol), and the other group was fed with a control diet that was replaced isocalorically with maltodextrin instead of ethanol, for a period of 7 weeks, and both were maintained and treated in accordance with the guidelines set by the Osaka Medical College for animal experimentation. They were killed with an overdose of pentobarbital administered by intravenous injection, and the spleens were used for the following studies.

Preparation of tissue sections

Small pieces of spleen were fixed in 10% buffered formalin for at least 24 h. After dehydration in a graded series of alcohol, they were embedded in paraffin, and serial paraffin sections (4–5 μm in thickness) were used for immunohistochemistry and the TUNEL method.

Some sections were stained with hematoxylin and eosin (H&E) to verify the structural integrity of the tissues.

Immunohistochemistry

The following antibodies were used: anti-rat p53 monoclonal antibody (Ab3; Oncogen, Cambridge, MA, USA), and antirat RM4 monoclonal antibody specific to macrophages (a gift from Professor M. Takeya, Dept. of Pathology, Kumamoto Medical and Pharmaceutical University, Kumamoto, Japan). In the case of p53 detection, a special microwave heating treatment was performed for antigen retrieval before adding the appropriate primary antibody. Subsequently, sections were stained using an indirect immunoperoxidase technique. Each primary antibody was used at a dilution of 1:100 with 0.01 M phosphate-buffered saline (PBS). After overnight incubation with the appropriate primary antibody at 4°C, sections were further incubated with the goat anti-mouse secondary antibody conjugated with horseradish peroxidase (DAKO, Glostrup, Denmark), diluted 1:100 with 0.01 M PBS, for 1 h. Peroxidase activity was analyzed by exposure of the sections to a solution containing 0.05% diaminobenzidine tetrahydrochloride and 0.01% H₂O₂ in Tris-HCl buffer at pH 7.6 for 5 min. Sections were finally counterstained with 1% methyl green and examined by light microscopy. Negative control experiments were performed using goat serum instead of primary antibodies.

The modified TUNEL method

The TUNEL method was based on the specific binding of TdT to free 3′-OH ends of DNA. In this modified TUNEL method, we used exogenous digoxigenin instead of endogenous biotin for the TUNEL method to prevent

Fig. 1A,B. Hematoxylin and eosin (H&E) staining for white pulp (WPs) of the control rats (A) and ethanol-treated rats (ETRs) (B). B WPs of ETRs show a “moth-eaten” appearance because of the marked depletion of splenocytes. Bar 70 μm