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Association of NKT cells and granulocytes with liver injury after reperfusion of the portal vein ☆

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Abstract

Reperfusion of the liver was conducted by clamping the portal vein for 30 min in mice, followed by unclamping. Unique variation in the number of lymphocytes was induced and liver injury occurred thereafter. The major expander cells in the liver were estimated to be natural killer T cells (i.e., NKT cells), whereas conventional T cells and NK cells increased only slightly or somewhat decreased in number and proportion at that time. Reflecting the expansion of NKT cells in the liver, a Th0-type of cytokine profile was detected in sera, and cytotoxic activity was enhanced in liver lymphocytes. In NKT cell-deficient mice including CD1d (−/−) mice and athymic nude mice, the magnitude of liver injury decreased up to 50% of that of control mice. It was also suspected that accumulating granulocytes which produce superoxides might be associated with liver injury after reperfusion. This might be due to stress-associated production of catecholamines. It is known that granulocytes bear surface adrenergic receptors and that they are activated by sympathetic nerve stimulation after stress. The present results therefore suggest that liver injury after reperfusion may be mainly caused by the activation of NKT cells and granulocytes, possibly by their cytotoxicity and superoxide production, respectively.

Keywords: NKT cells; Granulocytes; Liver injury; Reperfusion; Acute; Ischemia

1. Introduction

Organ reperfusion after acute ischemia is caused by various events, including trauma, surgical operation, and some types of inflammation. We empirically know that this reperfusion induces severe tissue damage, in addition to hypoxemia, due to some immune reactions of living body. Indeed, there have been several reports in which the accumulation of T cells in the liver was associated with this phenomenon [1–3]. Additionally, it is known that there are many kinds of lymphoid cells in the liver. Namely, the liver is the most abundant source of NK cells, extrathymic T cells and NKT cells among the immune organs [4–7]. Moreover, a number of conventional T and B cells which originate in the thymus and bone marrow are present [8,9]. In earlier studies on reperfusion [1–3], identification of these lymphocyte subsets was not achieved.

In light of these findings, in this study, we investigated how these lymphocyte subsets varied in proportion and number when reperfusion of the circulation was induced. Acute ischemia was produced by clamping of the portal vein in mice. Not only various lymphocyte subsets but also granulocytes were examined in this study. This idea comes from cumulating evidence that superoxides produced by granulocytes induce tissue damage even under aseptic conditions [10–13]. Both

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NKT cells and granulocytes were found to be intimately associated with liver injury after reperfusion.

It is well established that the liver ischemia induced by the clamping of whole vessels (including the artery and vein) at the hilum of a liver lobe evokes severe liver damage due to the activation of Kupffer cells [14–16]. In this experiment, we clamped only the portal vein to induce liver ischemia. This method induced lymphocyte accumulation in an area of the central vein and resulted in mild liver damage. This tissue damage therefore resembles that of clinical cases seen in humans. A major population of these lymphocytes was found to be NKT cells in the present study.

2. Materials and methods

2.1. Mice

C57BL/6 (B6), B6-*gld*/*gld* [17], perforin (−/−) (PKO)[1] [18], CD1d (−/−) [19,20] and B6-*nu*/*nu* mice were used at the age of 8–15 weeks. PKO and CD1d (−/−) mice were of B6 background. All mice were bred under specific pathogen-free conditions in the animal facility of Niigata University (Niigata, Japan).

2.2. Induction of ischemia in the liver

Mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal). The portal vein was then clamped for 30 min and then unclamped for reperfusion. This point of time is termed 0 h.

2.3. Cell preparation

Hepatic mononuclear cells (MNC) were isolated by a previously described method [21]. Briefly, the liver was removed, pressed through 200-gauge stainless-steel mesh, and suspended in Eagle's MEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5 mM Hepes and 2% heat-inactivated newborn calf serum. After being washed once with medium, the cells were fractionated by centrifugation in 15 ml of 35% Percoll solution (Amersham-Pharmacia Biotech, Piscataway, NJ) for 15 min at 2000 rpm. The pellet was resuspended in erythrocyte lysing solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA–Na, and 170 mM Tris, pH 7.3).

2.4. Immunofluorescence tests

FITC-, PE- or biotin-conjugated reagents of mAbs were used and biotin-conjugated reagents were developed with tricolor-conjugated streptavidin (Caltag Laboratories, San Francisco, CA) [22]. The mAbs used here were anti-CD3 (145-2C11), anti-IL-2Rβ (TM-β1), anti-NK1.1 (PK136), anti-macrophage (Mac-1), anti-granulocytes (Gr-1), anti-LFA-1, anti-CD69 and anti-CD28 mAbs (BD PharMingen, San Diego, CA). Cells were analyzed by FACScan (BD Biosciences, Mountain View, CA). To prevent nonspecific binding of mAbs, CD16/32 (2.4G2; BD PharMingen) was added before staining with labeled mAb. Dead cells were excluded by forward scatter, side scatter and propidium iodide gating.

2.5. In vivo injection of antibodies to eliminate lymphocyte subsets

To eliminate NK and NKT cells, anti-asialo GM_1 antibody (20 μl/mouse, Wako Pure Industries, Osaka, Japan) and anti-NK1.1 mAb (0.5 mg/mouse, BD Bioscience), respectively, were intraperitoneally injected 1 day before the experiments.

2.6. ELISA assay for the detection of IL-4 and IFNγ

Sera were used to detect the concentrations of IL-4 and IFNγ by ELISA assay using Opt EIA mouse IL-4 and IFNγ sets (BD PharMingen).

2.7. Measurement of transaminases

Liver injury was used to estimate the alanine aminotransferase (ALT) activity in sera. ALT activity in each supernatant was quantified with an STA TEST KIT (Wako Pure Industries).

2.8. Cytotoxic assay

Using YAC-1 targets, cytotoxicity was examined by specific ^{51}Cr-release assay with an incubation time of 4 h [23]. YAC-1 cells labeled with sodium chromate (^{51}Cr) (Amersham Int., Arlington Heights, IL) were used, and effector cells were hepatic MNC. Percent cytotoxicity was determined using 10^4 YAC-1 cells at the indicated target-to-effector ratios in triplicate cultures. During a 4-h incubation assay, spontaneous chromium release of both targets ranged from 10 to 15%. This release was eliminated by calculation.

2.9. Luminol-dependent chemiluminescence

Luminol-dependent chemiluminescence was determined as an indicator of superoxide production (more accurately, H_2O_2 and myeloperoxidase release) by a lumiphotometer (TD-4000; Labo Science, Tokyo, Japan). Superoxide production resulting from activation

[1] *Abbreviations used:* PKO, perforin knockout; MNC, mononuclear cells; NKT cells, natural killer T cells; $CD3^{int}$ cells, intermediate CD3 cells.