Protection of liver cells from experimentally-induced liver cell injury by cianidanol, a constituent of *Uncaria Gambir*

Yasuhiro MIZOGUCHI,*[a] Yoshihide SAKAGAMI,[a] Kenzo KOBAYASHI,[a] Sukeo YAMAMOTO[b] and Seiji MORISAWA[c]

[a] *The Third Department of Internal Medicine, Osaka City University Medical School*
[b] *The Osaka Socio Medical Center Hospital*
[c] *The First Department of Biochemistry, Osaka City University Medical School*

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Abstract

Liver cell injury is induced when isolated liver cells coated with specific antibody against the liver cell membrane are cultured with peripheral blood mononuclear cells. Although this antibody-dependent cell-mediated cytotoxicity (ADCC) is induced by closed contact of effector cells with targets via specific antibody, a cytotoxic factor or factors causing the inhibition of protein synthesis in liver cells are detected in the culture supernatant from the ADCC reaction. Similarly, peritoneal exudate macrophages activated by endotoxin lipopolysaccharide also have cytotoxic effects on isolated liver cells by producing a cytotoxic substance or substances. In this study, we found that liver cell injury caused by the ADCC and activated macrophage culture supernatant significantly improved when the isolated liver cells were treated with cianidanol before the addition of the cytotoxic culture supernatant. These results suggest that cianidanol may protect liver cells from immunological injury.

Key words antibody-dependent cell-mediated cytotoxicity (ADCC), activated macrophages, cianidanol, cell injury prevention

Abbreviations ADCC, antibody-dependent cell-mediated cytotoxicity ; EDTA, ethylenediamine tetraacetic acid ; HB, hepatitis B virus ; LPS, lipopolysaccharide ; LSP, liver specific lipoprotein ; MAF, macrophage activating factor ; MEM, minimum essential medium

Introduction

Autoantibodies which react with hepatocyte membrane lipoprotein complex (LSP) have been detected in sera obtained from patients with untreated chronic active hepatitis, and it has been reported that these autoantibodies may be involved in causing peripheral hepatocyte necrosis, which is characteristic of this condition.[1] Although it is possible that various immunological reactions may be induced against LSP, antibody-dependent cell-mediated cytotoxicity (ADCC) via anti-LSP antibody is considered to be related to the induction of liver cell injury.[2-4] The authors have previously demonstrated that liver cell injury due to ADCC is produced not only by closed contact between effector cells and tar-

*〒545 大阪市阿倍野区旭町 1-5-7
大阪市立大学医学部第三内科学教室 溝口靖紘
5-7, 1-chome, Asahi-machi, Abeno-ku, Osaka 545, Japan

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get liver cells via anti-LSP but also by some chemical mediators produced by the effector cells.[5] Furthermore, it has been suggested that liver cell injury may be induced by activated macrophages infiltrating into the liver tissue, because various lymphokines including the macrophage activating factor (MAF) are produced from LSP-sensitized T cells by stimulation with LSP and because the MAF-activated macrophages are presumed to participate in the induction of liver cell injury.[6] The authors have indicated that liver cell injury is induced not only by cell to cell contact between activated macrophages and target liver cells but also by the production of a cytotoxic factor or factors by the activated macrophages.[6,7]

Recent reports have suggested that cianidanol (chemical structure shown in Fig. 1),

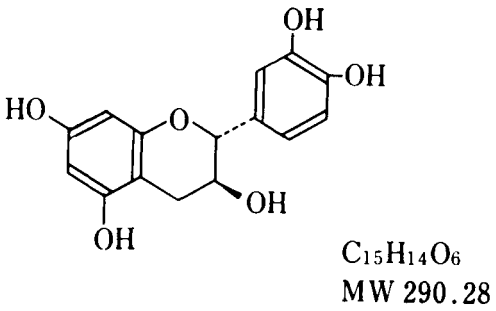

Fig. 1 Chemical structure of cianidanol.

a flavonoid compound extracted from *Uncaria Gambir*, may augment activities of mouse cytotoxic T-lymphocytes[8] and natural killer cells.[9] We have also reported that it stimulates lymphocyte transformation of peripheral blood lymphocytes obtained from patients with chronic active hepatitis and pokeweed mitogen-induced polyclonal antibody response.[10] Many experiments have shown that cianidanol affects the immune system,[8,9,11] and that it has free radical scavenger activity inhibiting lipid peroxide production[12,13] and a hepatoprotective effect on experimental liver injury caused by various toxic agents.[14-16] However, the functional mechanism of cianidanol on liver cell injury by immunological responses is still not clear. In the present study, we investigated the effects of cianidanol on liver cell injury induced by ADCC and activated macrophages.

Materials and Methods

Materials : Cianidanol was obtained from Zyma S. A. (Nyon, Switzerland). Eagle's MEM was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan) and fetal calf serum from M. A. Bioproducts (Maryland, U.S.A.). Liver specific lipoprotein (LSP) was prepared from the rat liver according to the method of McFarlane *et al.*[17] Liver homogenate in 0.25 M sucrose was centrifuges at 10500 *g* for 1 hr. Approximately 20 ml of the liver supernatant fraction was applied to a Sephadex G-100 column (2.5 × 90 cm) which had been previously equilibrated with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl and 1 mM EDTA. The column was eluted with the same buffer, and the first peak was concentrated to about 20 ml by ultrafiltration (diafilter G-10T, Bioengineering Co., Tokyo, Japan). Ten milliliters of this concentrate was applied to a Sepharose 6B column (2.5 × 90 cm), equilibrated and eluted with the same Tris/EDTA buffer. This first peak was concentrated as above, and the protein content was measured according to the method of Lowry *et al.*[18] Anti-LSP antibody was prepared as follows : Male rabbits weighing 3.5 kg were immunized by injections of LSP (10 mg protein) emulsified in an equal volume of Freund's complete adjuvant for four times at one week intervals. Seven days after the last booster injection, the immunized animals were sacrificed. The sera were then separated, and the complement was inactivated by heating at 56℃ for 30 min. The sera were stored at −70℃ until use.

Preparation of culture supernatant from ADCC reaction mixture : The liver cell suspension was prepared from normal rats according to the method of Bellemann *et al.*[19] After the cell concentration was adjusted to 1×10^5 cells/ml in Eagle's MEM containing 10% fetal calf serum, 0.01 ml/ml of antiserum was added to 1.0 ml of the isolated liver cell suspension and incubated at 37℃ for 3 hrs. To the antibody-coated he-

patocyte culture, 100 ml of lymphocyte-rich mononuclear cells prepared from the heparinized peripheral blood by Ficoll-Conray density gradient centrifugation (1×10^7 cells/ml) were added, and the cell mixture was incubated at 37℃ for 2 hrs in a humidified cell-incubator with aeration of 5 % CO_2 in air. After incubation, the culture supernatant was separated by centrifugation at 1000 *g* for 10 min.

Preparation of culture supernatant from LPS-activated macrophages : To obtain peripheral exudate cells mainly consisting of macrophages, 20 ml of sterilized Marcol 52 (Esso Oil Co.) was injected into the peritoneal cavity of normal guinea pigs. The peritoneal exudate cells were collected 4 days later by perfusing the peritoneal cavity with 200 ml of Hank's solution. After the oil phase was decanted, the aqueous phase was centrifuged at $800 \times g$ at 4℃ for 10 min. The pellets were washed three times with fresh Hank's solution and suspended in Eagle's MEM supplemented with 10% fetal calf serum to make a cell suspension of 5×10^6 cells/ml. LPS (Difco Co., Michigan, U.S.A.) was added to the peritoneal exudate cell suspension followed by incubation at 37℃ for 48 hrs. The culture supernatant was then separated by centrifugation.

Liver cell injury induced by culture supernatant from ADCC reaction mixture and activated macrophages and effects of cianidanol : The culture supernatant (0.5 ml) from the ADCC reaction mixture or activated macrophages prepared as described above was added to 0.5 ml of the liver cell suspension (1×10^6 cells / ml) freshly prepared from the normal rat liver. One μCi of [^{3}H]- L - leucine (specific activity 52 Ci/mmol, Amersham, UK) was then added followed by incubation at 37℃ for 24 hrs. After incubation, the radioactivity incorporated into the acid-insoluble fractions of the liver cells was measured with a liquid scintillation counter as reported previously.[5] Liver cell injury was evaluated by measuring protein synthesis. To measure the effects of cianidanol, the drug was diluted with Eagle's MEM and various concentrations were added to the liver cell suspension. After incubation at 37℃ for 6 hrs, the culture supernatant from the ADCC reaction mixture or activated macrophages was added, and protein synthesis in the liver cells was determined in the same manner as described above. Data were subjected to analysis of variance and t-tests with Bonferroni's correction. The level of significance was chosen at $p \leqq 0.05$.

Results

Effects of cianidanol on liver cell injury induced by culture supernatant from ADCC reaction mixture

When the culture supernatant separated from the ADCC reaction mixture was added to the freshly prepared liver cell suspension and liver cell injury was determined, it was found that the incorporation of [^{3}H-]-L-leucine into the protein was reduced to 57.7±5.5% of the control in which the culture supernatant from the ADCC reaction mixture was not added (Fig. 2). The

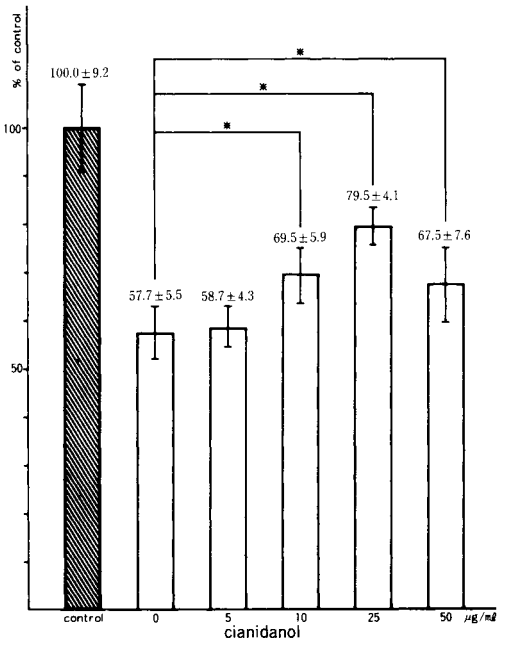

Fig. 2 Effects of cianidanol on liver cell injury induced by the culture supernatant from antibody-dependent cell-medicated hepatocytotoxic reaction mixture (*$p < 0.01$).
Liver cell injury was evaluated by the incorporation of [^{3}H]-L-leucine.

impaired protein synthesis in the liver cells was found to improve when the cells were pretreated with cianidanol. When the liver cells were pretreated with 5, 10, 25 and 50 μg/ml of cianidanol, protein synthesis in the liver cells was shown to be 58.7±4.3%, 69.5±5.9%, 79.5±4.1% and 67.5 ±7.6% of the control, respectively.

Effects of cianidanol on liver cell injury induced by culture supernatant from activated macrophages

When the cytotoxic activity in the culture supernatant from the activated macrophages was evaluated, the incorporation of [^{3}H]-L-leucine was 58.6 ± 9.8 % of the control as shown in Fig. 3.

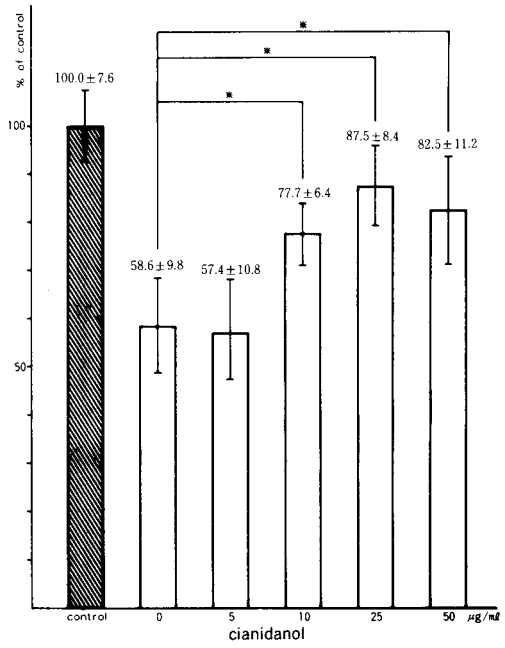

Fig. 3 Effects of cianidanol on liver cell injury induced by the culture supernatant from activated macrophages (*$p < 0.01$).
Liver cell injury was evaluated by the incorporation of [^{3}H]-L-leucine.

When the liver cells were pretreated with 5, 10, 25 and 50 μg/ml of cianidanol, protein synthesis in the liver cells was shown to be 57.4 ± 10.8%, 77.7 ± 6.4%, 87.5 ± 8.4% and 82.5 ± 11.2% of the control, respectively. These results indicated that the pretreatment with cianidanol protected the liver cells from damage by the activated macrophages as well as ADCC reaction. The most effective concentration of cianidanol was found to be 25 μg/ml in both cases.

Discussion

It has been well documented that cianidanol is beneficial in the therapy of HBe antigen positive chronic hepatitis.[20] A multi-centered, double blind trial has been performed to examine the efficacy of cianidanol in these patients. As a result, a significant decrease in HBe antigen titer was brought about in the cianidanol group compared to the placebo group, and the cases of seroconversion from HBe antigen-positive to the HBe antibody positive state were significantly higher in the cianidanol group. These results suggest that cianidanol may stimulate the immune system, because immune responses are considered to be responsible for the blood clearance of the virus. However, the detailed functional mechanism of cianidanol has not yet been completely clarified, although its action on the immune system,[8-10] free radical scavenger activity[12,13] and hepatoprotective effect on experimental liver injuries caused by various toxic agents[14-16] have been reported. In the present experiments, we confirmed the protective effects of cianidanol on experimentally-induced liver cell injury. It was clear from our previous experiments that the culture supernatant from either the ADCC reaction mixture or activated macrophages may contain cytotoxic substances which reduce protein synthesis in the liver cells.[5,6] Pretreatment of liver cells with cianidanol protected liver cells from such cytotoxic effects. Moreover, it was shown that the protective effects of cianidanol depended on the dose. The most effective concentration of the drug was 25 μg/ml, and larger amounts were not necessarily beneficial. Since the localization and metabolism of this drug in the liver cells are not clear, further investigations are necessary for the complete understanding of its mechanism.

和文抄録

正常ラット肝から分離調製した分離肝細胞を標的

細胞とし，これに抗肝細胞膜抗体を作用させ，次いで正常ヒト末梢血単核細胞をeffector細胞としてADCC反応を誘導し，その細胞培養上清を分離肝細胞に添加すると，肝細胞障害の指標として肝細胞におけるアルブミン合成が有意に低下した。またLPSで活性化した腹腔滲出マクロファージの培養上清を分離培養肝細胞に添加すると，肝細胞障害が発現して肝細胞のアルブミン合成が低下した。

しかし，前もってシアニダノールで処理した分離肝細胞に，前述のようにADCC反応細胞培養上清または活性化マクロファージ培養上清を添加培養すると，分離肝細胞におけるアルブミン合成の低下が有意に軽減することを認めた。以上の結果から，シアニダノールが，免疫性肝細胞障害を抑制する可能性があることが示唆された。

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