Discussions

Infection with HPV, the most important etiological agent for cervical cancer, does not manifest clinical symptoms. As HPV spreads along with other pathogens of STD, patients with complaints of lower genital tract infection requires to be examined as probable HPV carriers. The present study endeavored to clarify the relationship between STD and HPV infection in Japanese patients. The present study suggests patients with STD apparently have high risk of HPV infection and subsequent cervical cancer. On the contrary, results of our morphological study based on PAP smear and colposcopic biopsy samples showed a weak association between HPV infection and dysplastic changes. The possible combination of other factors such as genetic backgrounds including CYP2E1 GMTS1 and mEH polymorphism[9], smoking, local or systemic suppression of cellular immunity for developing cervical carcinogenesis required to be explored. Observing the presence of high risk HPV infection is not as sole indication for therapeutic procedures such as conization, LEEP or laser coagulation. However, these patients must be carefully followed up by cytological or histopathological examinations since past reports as well as the present study indicate that most of the cervical neoplasms are high risk HPV positive. Clustering of STD has been reported by many authors. Silins et al[10]. reported that significant clustering exists in cases of sero-positivity for HPV types 6 and 11, 16, 18, and 33 and for CT and HSV-2. Skapinyecz et al[11]. reported an increased prevalence of HPV infection in patients with PID. Though they have not commented on CT infection in their article, it has been reported that elsewhere CT is often associated with high risk HPV infection[12–14]. HPV and CT are the two most common sexually transmitted pathogens in Japan and other countries. CT infection has been regarded as a possible cofactor to HPV infection in the etiology of CIN diseases. Both have similar mode of transmission though CT infection possibly modulates the host's immunity and/or precipitation of chronic inflammation. As in the case with CT infection, HPV infection is also commonly assessed using detection of viral DNA in cervical epithelial cells. However, HPV shows a dynamic pattern with a very high rate of infection in the beginning years of sexual contact, followed by a high rate of spontaneous clearance: 70% of infections are cleared after 1 year[15, 16]. Therefore, studies on HPV infection based on the detection HPV DNA are inherently biased by the fact that they cannot distinguish between the initial phase of HPV acquisition and correlates of HPV clearance. In patients with CIN and invasive cervical neoplasia, some unknown events may exist that enable persistent infection and integration of HPV genomes. Thus some researchers recommend serological examinations instead of HPV DNA sampling. However, it should be kept in mind that serological tests only suggest history of past infection in uterine cervix and/or other organs and do not indicate current infection in the hot lesion of carcinogenesis. Hence, repeated sampling of HPV from uterine cervix in combination with PAP smears appears to be the most recommendable strategy. Many socioeconomic factors and behaviourl patterns are reported to be associated with acquisition of HPV. Numbers of sexual partners, early sexual debuts, parity, oral contraceptives, history of STD infection as well as smoking habits are considered to increase the risk of HPV infection and subsequent development cervical neoplasms. Of interest, we observed a statistically sig-nificant increase in smoking habit in HPV positive STD patients. Epidemiological studies also suggest about a possible role of cigarette smoking on carcinogenesis in the uterine cervix though no cause-effect relationship has been proposed. Harris et al. proposed a possible up-regulation of Ki-67 expression and subsequent increased cellular turnover at cervical squamo-columnar junction in smokers[17]. We suggest that the higher prevalence of HPV infection in the smoking STD patients result form persistence of infection due to suppression of local cellular immunity. Of interest, smoking has been reported to suppress NK activities[18, 19]. In conclusion, we observed clustering of high risk HPV and sexually transmitted infections though morpholpgical abnormalities were scarce. However our results suggest patients with STD requires regular follow up, including screening for HPV infection and cytological methods.

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Catechins prevents substance P-induced hyperactive bladder in rats via the downregulation of ICAM and ROS

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Abstract

We previously reported substance P (SP) via neurokinin type 1 receptor facilitates bladder afferent signaling and reactive oxygen species (ROS) formation in bladder connected with neurogenic inflammation [Am. J. Physiol. Renal Physiol. 284 (2003) F840]. Increased intercellular adhesion molecule expression and subsequent leukocyte adhesion in the inflamed bladder contribute to SP-induced oxidative injury. Here we investigate the effect of green tea extract (catechins) on SP-induced bladder hyperactivity. We evaluated isovolumetric cystometrogram, adhesion molecular expression, and ROS activity in anesthetized rat bladder with SP stimulation. Our results showed that SP increased the amount of leukocyte ROS production in vitro in a dose-dependent manner and the enhanced ROS can be inhibited by catechins treatment. Exogenous SP increased ROS in vivo in the bladder via increased intercellular adhesion molecule expression and subsequent leukocyte adhesion, a primary source of ROS in the inflamed bladder. Two weeks of catechins pretreatment reduced SP-induced bladder intercellular adhesion molecule expression and ROS amount and ameliorated the hyperactive bladder response. These results indicate that catechins pretreatment can ameliorate SP-induced neurogenic inflammation via the action of antioxidant, anti-adhesion, and anti-inflammatory activity.

Keywords: Reactive oxygen species; Hyperactive bladder; Substance P; Catechins; Adhesion molecule

The tachykinins substance P (SP) and neurokinins A and B belong to a family of neuropeptides that are distributed in the mammalian afferent nervous systems and commonly innervate smooth muscle, submucosal layers, and blood vessels of visceral organs [4,13,19]. On release from sensory afferents, SP, via neurokinin type 1 and 2 receptors, acts on smooth muscle or blood vessels to regulate visceral motility and blood flow [13]. Recent evidence suggests that tachykinins can induce visceral inflammation, and hyperreflexia [4,7,11] by increases in adhesion molecule expression, chemotaxis and activation of immune cells, mucus secretion, and water absorption/secretion in the lungs, gastrointestinal tract, and genitourinary tract [1,10].

The capsaicin-sensitive, SP-containing afferent fibers richly innervated the mammalian bladder [13] and regulated bladder efferent triggering micturition reflex [5,7]. Chien et al. [7] indicated that SP administration could initiate bladder hyperactivity by activating neurokinin receptors in both smooth muscle and sensory neurons and increase bladder afferent discharge to the central nervous system to result in a hyperactive bladder. Increased density of SP-containing fibers and substance P receptor-encoding mRNA appeared in the bladder with pathophysiological conditions, such as interstitial cystitis, cyclophosphamide-induced cystitis, and irritant-induced hypersensitivity [9,14,16]. Furthermore, increased SP via neurokinin type 1 receptor activation is known to increase adhesion molecule expression in the endothelial cells [15], cause generation of reactive oxygen species (ROS) by inflammatory cells [7] and, consequently, lead to a hyperactive bladder [1,7,13,20]. Attachment of

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leukocytes to the vascular endothelium and the subsequent migration of cells into the vessel wall are early events in the inflammatory course [3,12]. Since flavonoids (including catechins) differentially regulate IFN gamma-induced ICAM-1 expression in human keratinocytes [3,12], and catechins are also reputed to promote anti-inflammatory, and antioxidant activities [22], we investigated the possible effects of tea catechins on SP-induced neurogenic bladder inflammation.

Sixteen adult female Wistar rats weighing 220–250 g were used in this study. Groups of control rats ($n = 6$) and green tea extract treated rats ($n = 10$) were given water or green tea extract for 2 weeks. Fifteen grams of tea powder (Ten Ren Co., Ltd., Taipei, Taiwan) were soaked for 5 min in 500 ml of water at 100 °C and then filtered. The filtrate was freeze-dried, and the lyophilized powder was stored in a desiccator. The freeze-dried powder of green tea extract contained 249.9 mg/g of epigallocatechin gallate + epicatechin, 48.8 mg/g of epicatechin gallate, 61.8 mg/g of epigallocatechin, 2.8 mg/g of catechin, and 0.4 mg/g of gallic acid. The lyophilized powder (0.25 g) was dissolved in 500 ml of deionized distilled water every day. Each rat received restricted fresh drink (100 ml/kg body weight) daily provided at 6.00 p.m. in each metabolic cage by using sealed bottles for 2 weeks. For consistent dosage of catechins, the rest of tea drink in some rats was feed at 4.00 p.m. on the second day. The mean dosage of catechins was 50 ± 3 mg/kg body weight in this study.

After catechins pretreatment, rats were anesthetized with urethane (1.2 g/kg s.c.), which is well known to anesthetize the animals yet permit full reflex bladder contractions [7]. Maintenance of deep anesthesia was determined by the persistence of miotic pupils as judged from frequent inspection and by the lack of heart rate and arterial blood pressure (ABP) fluctuations in the absence of visceral stimuli [7]. An experiment was terminated when the baseline mean ABP was <90 mmHg. Animal care and experimental protocol were in accordance with the guidelines of the National Science Council of the Republic of China (1997). All efforts were made to minimize animal suffering and the number of animals used throughout the experiment. At the end of each experiment, the animals were killed by an intravenous potassium chloride injection.

PE-50 catheters were placed in the left femoral artery for measurement of ABP and in the left femoral vein for administration of potassium chlorite. ABP was recorded on a polygraph (model RS3400, Gould) with a transducer (model P231D, Gould-Statham, Quincy, MA). A length of stretched PE-10 tubing inserted just above the bifurcation of the aorta from the right femoral artery (i.a. route) was used for injection of SP. Body temperature was kept at 36.5–37 °C by an infrared light and was monitored with a rectal thermometer.

We used isovolumetric cystometrogram of the urinary bladder in this study [7]. We inserted one PE-50 tube into the bladder through the urethra and tied it in place with a ligature around the urethral orifice. The catheter was connected to a separate pressure transducer and an infusion pump via a T tube connector. Transurethral filling (0.15 ml/min) of 0.9% saline into the urinary bladder via the urethral catheter was done until rhythmic bladder contractions occurred. The infusion was stopped, and the bladder was maintained under constant-volume conditions by ligation of the ureter bilaterally. The bladder catheter was connected via a T tube to a pressure transducer (model P231D, Gould-Statham), and the intravesical pressure (IVP) was recorded continuously on a polygraph (model RS3400, Gould, Cleveland, OH). The number of active contractions (>15 mmHg) was evaluated during each 10-min interval. The change in IVP, ABP, and various parameters measured by the cystometrogram can be determined before and after intra-arterial administration of SP. SP was injected through the intra-arterial catheter in a volume of 1 ml/kg (0.20–0.25 ml) and was followed by 0.1 ml of heparinized saline. We evaluated the effect of exogenous SP on bladder hyperactivity. SP was given at 10 μg i.a. [7].

SP is capable of causing bladder ROS generation by inflammatory cells [7] and thus, contributes to bladder hyperactivity. The ROS generation in response to SP stimulation was measured in leukocytes and bladder by a chemiluminescence (CL) detection method as described previously [6]. The method for detection of ROS from the organ surface after 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-(1,2-*a*)-pyrazin-3-one hydrochloride administration (MCLA, 0.2 mg/ml/h i.a.) was adapted from the technique first described by Chien et al. [6] and was modified for demonstration of ROS production in the hyperactive bladder [7]. The MCLA-enhanced CL signal from the bladder surface was measured continuously before and during SP administration by use of a CL analyzing system (CLD-110, Tohoku Electronic Industrial, Sendai, Japan). A portion of the urinary bladder was cut and fixed in 10% neutral buffered formalin solution, dehydrated in graded ethanol, and embedded in paraffin. Sections (5 μm) of bladders were stained with hematoxylin and eosin for evaluation of the extent of leukocytes accumulation [7].

Before SP stimulation, baseline ABP, IVP, and ROS amounts were recorded for 30 min as a control value. Post SP injection, the ABP, bladder responsiveness and ROS of two groups of rats were evaluated. The ABP and cystometrogram were monitored simultaneously for 60 min. After measurement, the bladder tissues were collected and stored at −70 °C for immunoblotting analysis. The immunoblotting method was described previously [6,7]. We measured the amounts of ICAM and β-actin in bladder tissues. For protein analysis, bladder samples were homogenized with a prechilled mortar and pestle in extraction buffer, which consisted of 10 mM Tris–HCl (pH 7.6), 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40, 0.5% deoxycholate, 2% β-mercaptoethanol, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin. The homogenate was centrifuged at 12,000 × *g* for 12 min at 4 °C, the supernatant was collected, and protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules,

CA). Antibodies raised against ICAM (catalog no. AF583, R&D Systems, Minneapolis, MN) and β-actin (catalog no. A5316, clone AC-74, Sigma) were used. Both of these antibodies cross-react with respective rat antigens.

SDS–PAGE was performed on 12.5% separation gels in the absence of urea, and the gels were stained with Coomassie brilliant blue. Proteins on the SDS–PAGE gels, each lane containing 30 μg of total protein, were transferred to nitrocellulose filters. The immunoreactive bands were detected by incubation with the antibody described above, the secondary antibody-alkaline phosphatase, and, finally, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche Diagnostic, Mannheim, Germany) stock solution for 30 min at room temperature. Values are displayed as means ± S.E.M. Data were subjected to analysis of variance, followed by Duncan's multiple-range test for assessment of the differences among groups. Student's paired *t*-test was used to detect differences between control and drug treatment. $P < 0.05$ was considered to indicate statistical significance.

The SP-treated leukocytes showed a dose-dependent increase in ROS formation: 102 ± 19 counts/10 s, 360 ± 39 counts/10 s, 580 ± 59 counts/10 s, 878 ± 120 counts/10 s at 0, 1, 5, and 10 μg of SP, respectively (Fig. 1). Coincubation with the catechins (from 0.1 to 1 μg) reduced SP-induced ROS generation from leukocytes, indicating catechins' antioxidant activity.

In the isovolumetric condition without SP stimulation, the frequency of bladder contractions was 3.0 ± 0.3 active contractions/10 min, and the basal level of bladder ROS was maintained around 120 ± 20 counts/10 s in the control group. Intra-arterial SP stimulation significantly reduced ABP (from 105 ± 4 mmHg to 70 ± 5 mmHg, $P < 0.05$) and increased the frequency of active contractions (8.8 ± 1.4 active contractions/10 min, $P < 0.05$) and the bladder ROS generation (Fig. 2, left panel; from 120 ± 20 counts/10 s before SP stimulation to 2600 ± 250 counts/10 s during 30 min of SP, $P < 0.05$). The response of the hyperactive bladder and the increase in ROS generation could be maintained for >30 min. Pretreatment of catechins is potentially used to prevent and/or reduce SP-induced hyperactivity (Fig. 2, right panel; from 3.1 ± 0.4 active contractions/10 min to 3.8 ± 0.6 active contractions/10 min, $P = 0.35$) and ROS generation from the bladder surface (from 130 ± 22 counts/10 s to 1160 counts/10 s, $P < 0.05$). These data showed that catechins pretreatment could ameliorate SP-induced bladder hyperactivity and oxidative stress.

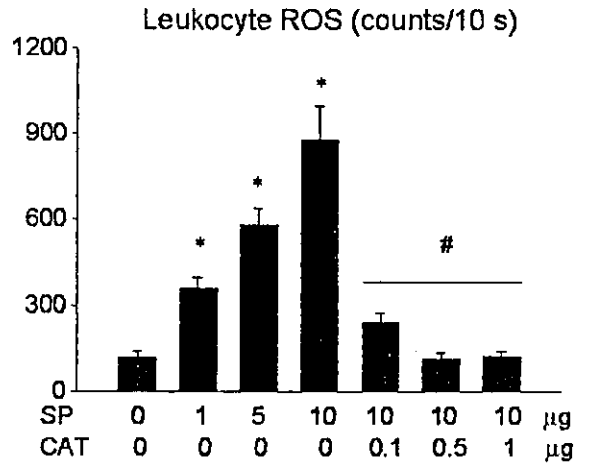

Fig. 1. SP-induced reactive oxygen species (ROS) generation in leukocytes. 0–10 μg of SP-induced leukocyte ROS formation in a dose-dependent manner. Effect of 10 μg of SP can be prevented by coincubation with catechins (CATS). * $P < 0.05$ vs. control. # $P < 0.05$ vs. SP without CATS treatment.

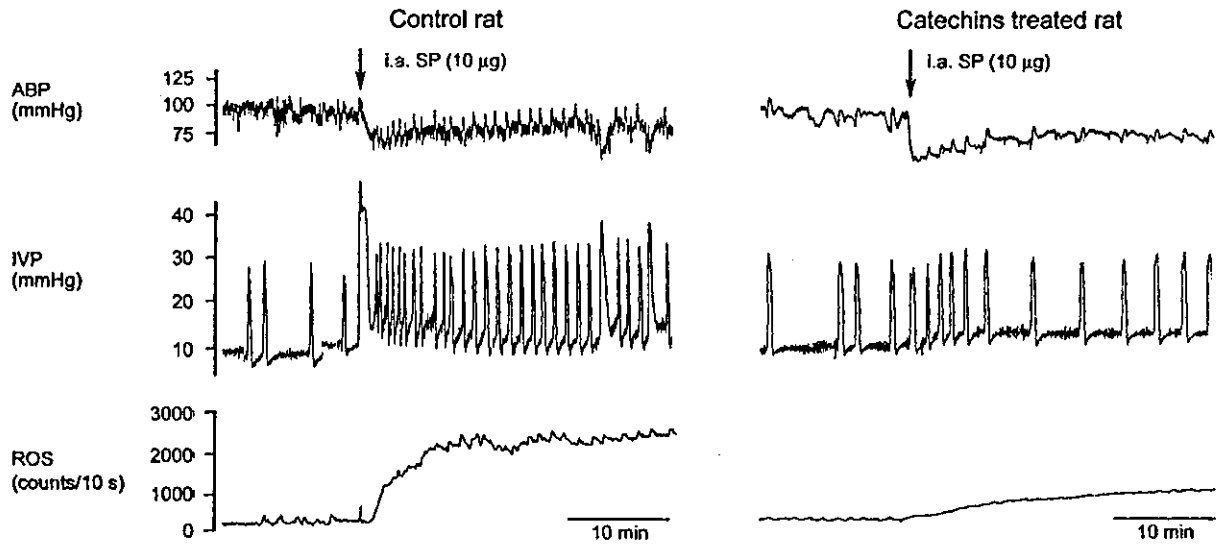

Fig. 2. In vivo response of SP-induced bladder hyperactivity and bladder ROS generation in an isovolumetric model. Intra-arterial SP injection induced significant vasodilation and bladder hyperactivity, as well as bladder ROS generation (left panel). Pretreatment with catechins reduced the frequency of bladder contractions and decreased, in part, ROS generation in the bladder (right panel).

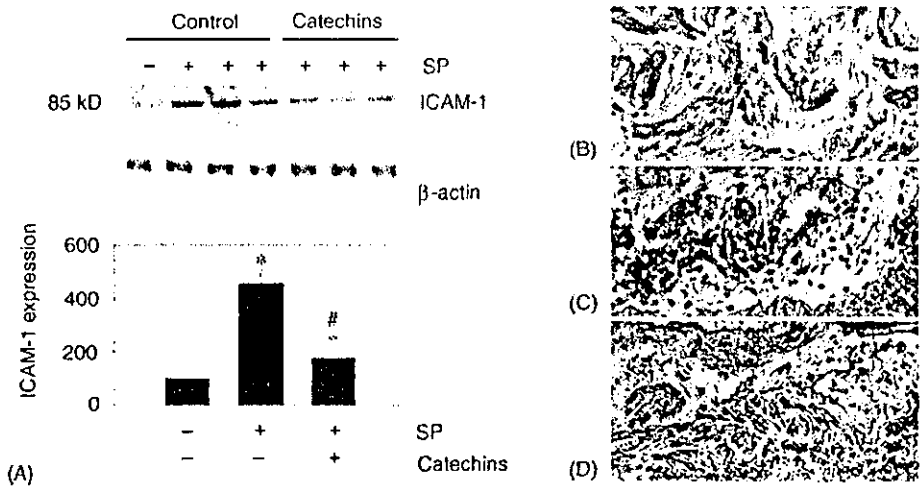

Fig. 3. ICAM expression and leukocytes in the control (A) and SP-induced hyperactive bladder (C and D). Intra-arterial SP (10 μg) stimulation increased expression of ICAM (85 kDa) in the bladder tissue. The expression of ICAM appeared to be decreased by pretreatment of catechins (500 μg). Equal protein loading was displayed by β-actin. Lower panel is the mean data by densitometry. Leukocytes primarily occurred in the vicinity of the blood vessels in the SP-treated bladder (C), but not in the control bladder (B). Note that the decreased number in leukocytes adhered to the endothelium in one SP rat pretreated with catechins (D).

Expression of ICAM and β-actin in the bladder after SP treatment was assessed by immunoblotting with antibodies against ICAM and β-actin (Fig. 3A). ICAM expression was detected in control bladder. ICAM expression in bladder tissues was significantly increased 60 min post SP stimulation. However, the enhanced ICAM expression was significantly downregulated by catechins (4.7 ± 0.5-fold in control versus 1.8 ± 0.3-fold in catechin treatment, $P < 0.05$). Sections from the bladder tissue obtained 60 min after SP administration revealed increased infiltration of leukocytes (Fig. 3C). Sections of urinary bladders from control animals without SP stimulation (Fig. 3B) and catechins treated animals with SP stimulation (Fig. 3D) exhibited no significant signs of leukocyte infiltration at the end of the experiments.

The present study demonstrates the involvement of leukocyte-ROS generation in SP-mediated bladder hyperreflexia. Stimulation by exogenous SP can enhance central and peripheral afferent signaling, sensitize micturition reflex, and lead to bladder hyperactivity [7,9,13]. Recently, we have demonstrated that i.a. SP through neurokinin type 1 receptors could initiate hyperactive bladder and increased mast cell degranulation and leukocyte dependent ROS generation [7]. Additionally, the release of SP determines a set of responses (loosely defined as neurogenic inflammation) that includes vasodilatation, plasma protein extravasation, smooth muscle contraction, stimulation of afferents, and inflammation [7,8]. To further complicate matters, sensory neurons and immune cells can express and release tachykinins, which may also contribute to neurogenic inflammation in the bladder. SP is able to induce secretion of various cytokines (e.g., interleukins-1 and -6 and tumor necrosis factor-α) from cultured lymphocyte-enriched mononuclear cells isolated from human peripheral blood [8]. SP has also been shown to cause a proinflammatory changes, such as degranulation of mast cells and leukocyte adhesion [7], by enhancement of ICAM expression in the bladder tissue (Fig. 3A). Saban et al. [17] have confirmed the role of SP in induction of bladder inflammation by the evidence that a dramatic reduction in antigen-induced cystitis in neurokinin type 1-receptor-deficient mice was found.

As a consequence of SP-immune and -inflammatory cell interaction, a variety of substances, such as histamine, cytokines, and ROS, are released [4,8,17,18,21]. In leukocytes incubated with SP, ROS activity is displayed in a dose-dependent manner and SP-induced ROS release is greatly inhibited by catechins, confirming a downregulating effect on oxidative stress by catechins. Catechins have been shown to inhibit histamine release from mast cells through inhibiting tyrosine phosphorylation of proteins (focal adhesion kinase 125) [22]. We further showed that SP-induced ROS generation in the bladder as well as bladder hyperreflexia was markedly inhibited by catechins. ROS are known to affect muscle tone, vascular smooth muscle strip contraction [2,7], and increased neural activity/conduction velocity in vitro by mechanisms such as alterations in membrane conductance, calcium homeostasis, calcium-dependent processes, and eicosanoid and nitric oxide metabolism [2]. Furthermore, increased SP via neurokinin type 1 receptor activation is known to increase ICAM in the endothelial cells, cause generation of ROS by inflammatory cells [7] and, consequently, lead to a hyperactive bladder [1,7,13,20]. Attachment of leukocytes to the vascular endothelium by an upregulation of ICAM is one potentially early event in the inflammatory course [3,12]. Our data indicate that catechins downregulate ICAM-1 expression in the SP-infused bladder tissues.

In summary, our studies provide evidence that SP evokes hyperactive bladder afferent signaling via the immunomod-

ulatory action of leukocyte ROS production and ICAM expression. Daily pretreatment of catechins may prevent SP-induced bladder hyperactivity by the downregulation of ROS formation and ICAM expression, indicating a therapeutic potential in the treatment of hyperactive bladder.

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Granulocyte Colony-stimulating Factor Suppresses Autologous Tumor Killing Activity of the Peripheral Blood Lymphocytes in the Patients with Ovarian Carcinoma

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PROBLEM: Granulocyte colony-stimulating factor (G-CSF) is often administered to patients with chemotherapy-induced leukocytopenia. However, adequate attention has not been paid to its effects on cancer immunology. Reported by us and others, G-CSF often induces immunosuppression and down-regulation of response T helper (Th)2 directed immune reaction both *in vivo* and *in vitro*. In this study, we analyzed the effects of G-CSF on interferon (IFN)-γ production and autologous tumor killing (ATK) activities of peripheral blood mononuclear cells (PBMCs).
METHODS OF STUDY: In order to evaluate the cytokine-induced activation of peripheral T and natural killer (NK) cells, we analyzed IFN-γ production by interleukin (IL)-2- and IL-12-stimulated PBMCs, using the ELISPOT assay. Specific killing of autologous tumor cells was evaluated by lactate dehydrogenase (LDH) release assay.
RESULTS: The PBMC collected from both cancer-bearing patients and healthy subjects showed IL-2- and/or IL-12-induced IFN-γ production. The frequency of IFN-γ producing cells was significantly higher in the normal subjects compared with the patients with advanced ovarian carcinoma. The ATK activity was also enhanced in IL-2- and/or IL-12-stimulated PBMCs of patients with ovarian carcinoma. G-CSF almost completely abolished IFN-γ production and ATK activity of PBMC stimulated with IL-2 and/or IL-12.
CONCLUSIONS: The G-CSF appears to be a suppressor of antitumor immunity. Routine administration of G-CSF to cancer patients may not be recommended, except for febrile neutropenia.

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INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is one of the most commonly used cytokines for chemotherapy-induced leukocytopenia and peripheral blood stem cell transplantation.[1,2] G-CSF is a glycoprotein consisting of 174 amino acids with a molecular weight of 18–22 kDa (depending on the degree of glycosylation). It acts through mobilization of stem/progenitor cell from bone marrow to peripherals as well as inducing differentiation of myeloid cells. In physiologic conditions, G-CSF is produced by macrophages, monocytes and endothelial cells.

Recent studies suggest a broad immunomodulatory role of G-CSF apart from its highly selective hematopoietic effects.[3–5]

We have recently reported on its roles in reproductive immunology.[6] Physiologic leukocytosis observed during the second and third trimesters of pregnancy is attributed to the local production of G-CSF and macrophage (M)-CSF in placental tissue. We reported that G-CSF abolished interferon (IFN)-γ production and cytotoxicity of decidual lymphocytes activated with interleukin (IL)-2 and/or IL-12 against choriocarcinoma cell lines and primary culture trophoblasts.[6,7]

Changes in immune responses during pregnancy and cancer-bearing conditions often share common futures, such as local and systemic T helper (Th)2 predominance[8–10] and local accumulation of suppressive γδ T-lymphocytes.[11] We assumed that G-CSF can be another candidate for immune suppression, because unfavorable prognosis has been reported in G-CSF producing malignancies.[12,13]

Probably, G-CSF may also suppress antineoplastic immune response of cancer-bearing hosts. In this study, we undertook the present study to examine the potential of G-CSF in suppression of antitumor immune response of peripheral blood lymphocytes against autologous ovarian carcinoma cells.

MATERIALS AND METHODS

Primary Culture of Ovarian Carcinoma Cells

Eight patients with advanced primary epithelial ovarian carcinoma comprised the study group. Informed consent was obtained from all patients before sample collection. No patients received chemotherapy, hormone therapy, and immunotherapy or radiation therapy before sampling. Carcinoma cells were collected from ascitic fluid during laparotomy or by abdominocentesis.

Primary culture of carcinoma cells were established as follows. Ascitic fluid samples were centrifuged at $2000 \times g$ for 10 min. The pellet was washed twice with phosphate-buffered saline [PBS (−), pH 7.4 WakoJunyaku, Tokyo, Japan], resuspended in RPMI FCS10% 1640, and layered onto Lymphocyte Separation Medium (LSM; ICN Biomedicals, Inc., Aurora, OH, USA) then centrifuged at $1500 \times g$ for 30 min. The cells at the interface were washed twice with PBS and re-suspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS). The cells were then plated into culture bottles and were cultured at 37°C in a continuous flow of 95% air, 5% CO_2.

Preparation of Peripheral Blood Mononuclear Cell

Peripheral blood mononuclear cells (PBMCs) were prepared from samples obtained from eight patients with advanced ovarian carcinoma and 11 age-matched healthy subjects, as described previously.[7]

Briefly, heparinized blood samples obtained by venipuncture were layered onto LSM (ICN Biomedicals, Inc.) then centrifuged at $600 \times g$ for 30 min to remove red blood cells and cell debris. The cells at the interface were washed twice with PBS and resuspended in RPMI 1640 supplemented with 10% FCS. CD4-, CD8- and CD16-positive cells were separated from PBMC using Magnetic Cell Sorting and Separation System (MACS; Dai-ichi Kagaku, Tokyo, Japan) according to the manufacturer's manual.[14] Briefly for separation of CD8-positive cells, PBMC were incubated with anti-CD8 monoclonal antibody-coated immunomagnetic beads (AF12-7H3.6.11; Miltenyi Biotec, Bergisch Gladbach, Germany; Dai-ichi Kagaku) for 45 min at 4°C. Cells unbound to magnetic beads were gently separated from bound cells through magnetic column. Cells bound to magnetic beads were used as $CD8^+$ enriched fraction. By the same way CD4- and CD16-positive cells were separated with microbeads coated with the corresponding monoclonal antibody (Miltenyi Biotec).

Cytokines

The IL-2 was kindly provided by Shionogi Pharmaceutical Co. Ltd (Osaka, Japan) as a purified recombinant protein with a specific activity of 5×10^6 IU/mg. We purchased human recombinant IL-12 from Genzyme-Cosmobio (Tokyo, Japan) and G-CSF (Pepro Tech EC, Rocky Hill, NJ, USA). Their specific activities were 1×10^6 IU/mg and 1×10^5 IU/mg, respectively.

ELISPOT Assay for IFN-γ Producing Cells

In order to examine the number of IFN-γ producing cells in MACS-enriched PBMC, the cytokine-specific ELISPOT assay was employed using the commercially available ELISPOT assay system (Mabtech AB, Nacka, Sweden). Details of this assay have been described previously.[6,15] Briefly, a 96-well plate with nitrocellulose base (Mititer HA; Millipore, Bedford, MA, USA) was coated overnight at 4°C with 100 μL/well of 8 μg/mL mouse antihuman IFN-γ capture monoclonal antibody by overnight incubation at 4°C (9D7; Mabtech). Decidual mononuclear cells (DMNCs) as well as MACS-enriched CD8-positive decidual lymphocytes (1×10^3–1×10^4 cells/well) were added to each well.

After 20 hr of culture, plates were washed and incubated with a biotinylated anti-IFN-γ second mouse monoclonal antibody (12G8; Mabtech, 1 μg/mL), followed by treatment with avidine-peroxidase and the substrate BCIP/NBT (Bio-Rad, Richmond, CA, USA). After overnight drying at room temperature, colored spots were counted using a microscope

equipped with an automated KS ELISPOT analysis system (Carl Zeiss Japan, Tokyo, Japan)

Reverse Transcription Polymerase Chain Reaction for Detection of G-CSF Receptor Expression

The RNA was extracted from freshly prepared samples and MACS-enriched PBMC by the AGPC method using an RNAzol™ (Biotecx, Houston, TX, USA) rapid RNA purification kit. The total RNA solutions were adjusted to a concentration of 1 μg/μL with diethyl pyrocarbonate (DEPC)-treated water and stored at −70°C until use.

Single-strand cDNA copy was made from 1 μg of total RNA, employing random hexamers and reverse transcriptase (first strand cDNA synthesis kit; Pharmacia, Tokyo, Japan), as follows. After incubation at 65°C for 10 min, reverse transcription was performed at 37°C for 1 hr.

Amplification was performed using 0.2 U of AmpliTaq (Perkin-Elmer, Cetus Tokyo, Japan) in 2.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, 0.01% gelatin (w/v) supplemented with 0.25 mM each of dATP, dGTP, dCTP and dTTP and 1 pmol of each primer, by a pre-cycle at 96°C 180 s following 35 cycles of 60 s at 96°C, 60 s at 52°C, and 120 s at 72°C in a thermal cycler (Perkin-Elmer).

The primer pair used for this study was 5′-CCA AGA GCA GTT TCC ACC CAG GCC-3′ for sense, and 5′-GTA GAT CTT AGT CAT GGG CTT ATG G-3′ for antisense strands, according to Brandstetter.[16]

After the final cycle, samples were kept at 72°C for 10 min in order to complete the synthesis of the polymerase chain reaction (PCR) products. Specificity of the PCR product was verified by electrophoresis in 2% agarose gel followed by direct sequencing.

Cytotoxicity Assays

Roughly prepared and MACS-sorted $CD4^+$, $CD8^+$ T cell and $CD16^+$ natural killer (NK) cell were cultured for 48 hr in presence of IL-2 (100 IU/mL), IL-12 (10 IU/mL) and different concentrations of G-CSF(10 or 100 pg/mL). Cytotoxic activity of IL-2/IL-12-activated cells was evaluated using lactate dehydrogenase (LDH) release assays.[16] Briefly, 96-well round-bottomed microwel plates were prepared with 100 μL triplicate serial dilutions of cytokine-activated PMC in effecter cell; target cell concentrations of 30:1.

Activated PMCs were washed twice with PBS and suspended in serum-free S-clon media (Sanko Co., Tokyo, Japan), in order to remove indigenous LDH than in FCS. Four hours after incubation at 37°C in 5% CO_2, 100 μL of supernatant was transferred to the corresponding wells of a flat-bottomed microtiter plate. Subsequently, 100 μL of lactic acid dehydrogenase mixture [5.4×10^{-2} M (+) lactate, 6.6×10^{-4} M 2-*p*-iodophenyl-3-*p*-nitrophenyl tetrazolium chloride, 2.8×10^{-4} M phenazine methosulfate and 1.3×10^{-3} M nicotinamide adenine dinucleotide (NAD) in 0.2 M Tris-Hcl pH 8.2] was added.

After 5 min incubation at 37°C, we examined absorbance at 490 nm on an automated optical microplate reader (MPRA4-I-II Toso, Tokyo, Japan). We examined linearity of the optical density with serially diluted LDH controls and accordingly compensated for the spontaneous release as reported by Konjevic.[17] The cytotoxicity was calculated according to the following formula: % cytotoxity = sample LDH release − spontaneous LDH release (by untreated target cells + effectors cells)/maximum LDH value obtained by total cell lyses − spontaneous LDH release.[18]

RESULTS

Expression of G-CSF Receptor mRNA of MACS-sorted Peripheral Mononuclear Cells

First, we examined the expression of G-CSF receptor (G-CSFR) mRNA in antigenically separated peripheral lymphocytes by reverse transcription polymerase chain reaction (RT-PCR).

Then, we observed ubiquitous expression of G-CSFR mRNA in unsorted PBMC, $CD4^+$, $CD8^+$ T cells and $CD16^+$ NK cells (Fig. 1).

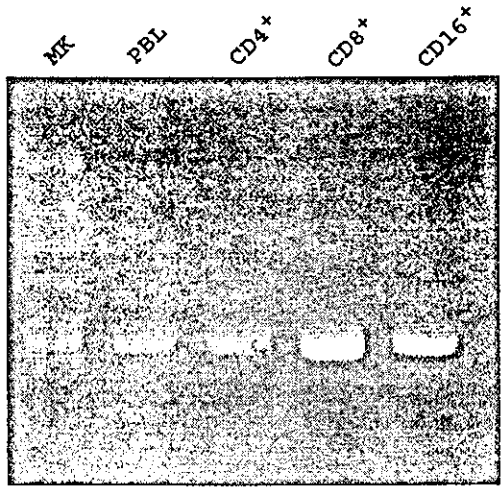

Fig. 1. Reverse transcription polymerase chain reaction (RT-PCR) analysis of granulocyte colony-stimulating factor (G-CSF) mRNA in Magnetic Cell Sorting and Separation System (MACS)-sorted peripheral blood lymphocytes (PBL). Total PBL (lane 1), $CD4^+$ (lane 2), $CD8^+$ (lane 3), $CD16^+$ (lane 4) were examined for expression of G-CSF receptor mRNA. Positive signal of 470 bp was observed in all samples examined.

IL-2/IL-12-stimulated Production of IFN-γ in PBMCs is Suppressed by G-CSF

The frequency of IFN-γ producing cells was measured by ELISPOT assay. Neither unfractionised PBMCs from healthy control nor MACS-separated $CD4^+$, $CD8^+$ T cells and $CD16^+$ NK cells from patient samples produced detectable levels of IFN-γ insignificant numbers, after 48 hr culture. However, by IL-2 and/or IL-12 stimulation, the number of IFN-γ producing cells increased up to 10–100 cells/10^4 cells. The strongest induction was observed in cells stimulated with both IL-2 and IL-12 at the same time. Simultaneous administration of G-CSF (100 pg/mL) almost completely abolished IL-2/IL-12-induced IFN-γ production (Figs 2 and 3).

A statistically significant suppression of IFN-γ production was observed in the samples collected from the patients with ovarian carcinoma, compared with the samples of healthy subjects.

G-CSF Suppresses IL-2/IL-12-induced ATK Activity of PBMCs without Affecting their Viability

Peripheral blood mononuclear cells were cultured for 48 hr in the presence or absence of IL-2 (100 IU/mL) and/or IL-12 (10 IU/mL). Primary culture of carcinoma cells was established from ascites fluid samples as targets. We estimated autologous tumor killing (ATK) activity by tumor cell/activated lymphocyte coculture experiments at effecter/target (E/T) ratio of 30:1.

Morphologically, we observed degeneration of ovarian cells cultured with IL-2- and IL-12-activated PBMCs although the sheet-like structure of carcinoma cells was conserved in the culture with PBMCs treated with IL-2, IL-12 and G-CSF (Fig. 4).

Using LDH release assay, we observed a dose-dependent suppression of the cytotoxicity of IL-2- and/or IL-12-activated peripheral blood lymphocytes by G-CSF over 100 pg/mL. Simultaneous treatment of PBMC with G-CSF in combination with IL-2/IL-12 suppressed the cytotoxicity of PBMC in a dose-dependent manner (Fig. 5).

This suppression could not be attributed to a direct cytotoxic effect of G-CSF on peripheral lymphocytes because 48 hr culture of MACS-sorted PBMC was not affected by G-CSF from range of 10 to 200 pg/mL (Fig. 6).

DISCUSSION

The G-CSF is administered to patients with leukocytopenia and peripheral blood stem cell transplantation. For gynecologic as well as other chemotherapy-sensitive malignancies, some clinicians recommend routine G-CSF administration, in order to increase the dose intensity of chemotherapeutic agents including platinum and taxoids.[19,20] However, recent reports suggesting that G-CSF suppresses NK and cytotoxic T lymphocyte (CTL) activity aroused our awareness its possible role in suppression of antitumor activity. Because, Zang et al. revealed recently that a strong correlation exists between the degree of tumor infiltrating lymphocytes and the progression-free survival as well as overall survival of the patients with advanced ovarian carcinoma. In other words, host immune responses affected prognosis of the patients with epithelial ovarian carcinoma.[21]

For the analysis of cellular immune responses in cancer-bearing patients, most of the past reports had employed established malignant cell lines such as K562 and Daudi as NK and lymphokine-activated killer (LAK) targets respectively. In contrast, we analyzed specific cytotoxicity by the ATK assay, employing primary culture carcinoma cells instead of established cell lines.

Proposed by Uchida in 1994, ATK assay is considered to be the most reliable assay method for the reflection of antitumor immune response *in vivo*.[22,23] The strong correlation between ATK activity and

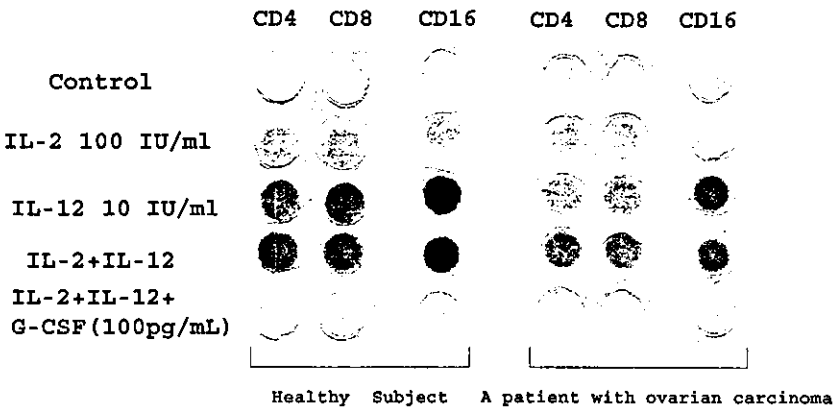

Fig. 2. Representative ELISPOT patterns of interferon (IFN)-γ production of a healthy subject and a patient with ovarian carcinoma. Peripheral lymphocytes were sorted into $CD4^+$, $CD8^+$ and $CD16^+$ cells. Cells were cultured in the presence of interleukin (IL)-2 and/or IL-12 with/without granulocyte colony-stimulating factor (G-CSF) for 24 hr. Purple spot shows IFN-γ-producing cells. A spontaneous IFN-γ production was not observed in a healthy subject or a patient with ovarian carcinoma. IL-2- and/or IL-12-induced IFN-γ in both samples while 100 pg/mL of G-CSF completely abrogated IFN-γ induction.

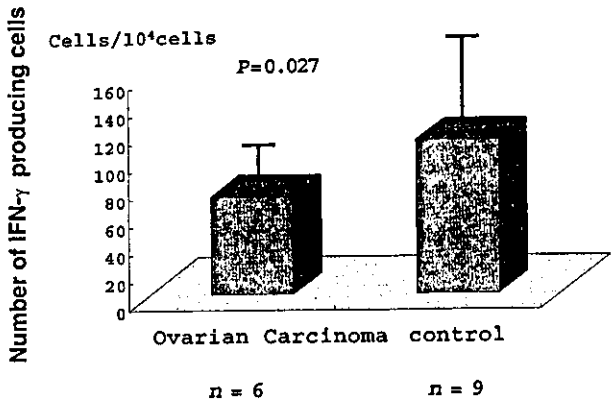

Fig. 3. Cumulative numbers of the interferon (IFN)-γ-producing peripheral lymphocytes. The numbers of IFN-γ-producing peripheral lymphocytes stimulated with interleukin (IL)-2 100 IU and IL-12 10 IU was examined by ELISPOT assay in nine healthy subjects and six patients with advanced ovarian carcinoma. Fewer number of IFN-γ-producing cells were observed in the patients with ovarian carcinoma. The difference was statistically significant.

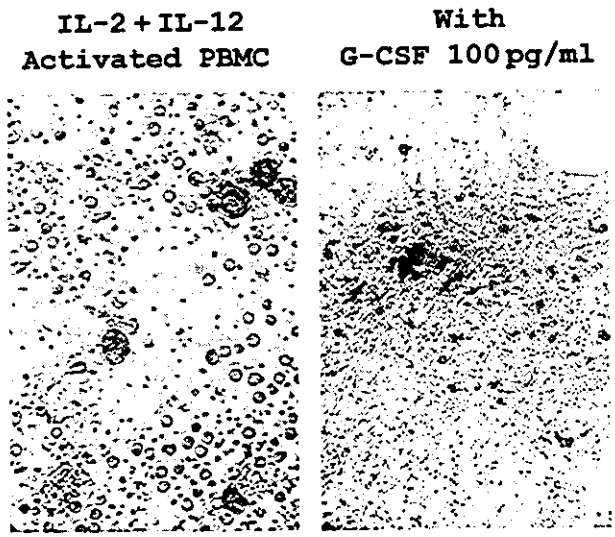

Fig. 4. Granulocyte colony-stimulating factor (G-CSF)-induced suppression of autologous tumor killing activity of interleukin (IL)-2/IL-12-stimulated peripheral blood mononuclear cells (PBMC). PBMCs were cultured for 48 hr in the presence of IL-2 (100 IU/mL) and IL-12 (10 IU/mL). Primary culture of ovarian carcinoma was established from an ascites samples. We cultured tumor cells and lymphocytes by effecter/target (E/T) ratio of 30:1. Remarkable morphologic changes in ovarian carcinoma cells cultured with IL-2- and IL-12-activated PBMC (left) while simultaneous administration of G-CSF (100 pg/mL) for PBMC strongly inhibited their cytotoxicity (right).

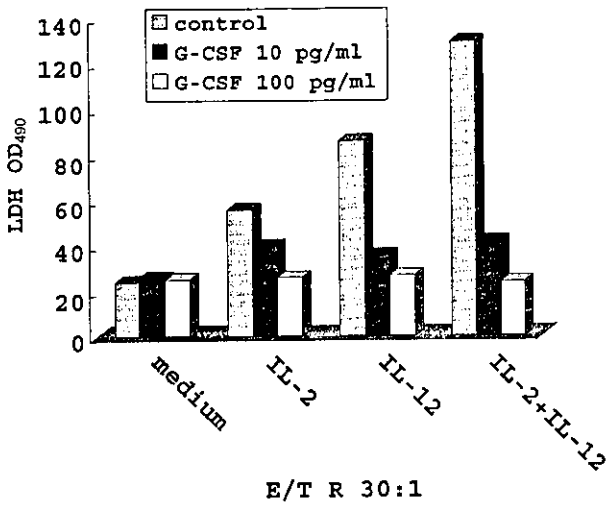

Fig. 5. Granulocyte colony-stimulating factor (G-CSF) suppresses interleukin (IL)-2/IL-12-induced autologous tumor killing activity of peripheral lymphocytes. Effect of G-CSF for autologous tumor killing (ATK) was examined with lactate dehydrogenase (LDH) release assay. Primary culture of ovarian carcinoma cells was established from an ascites sample. Peripheral blood mononuclear cells were cultured for 48 hr in the presence of IL-2 (100 IU/mL) and IL-12 (10 IU/mL). After tumor and lymphocytes culture (MTLC) were mixed (E/T rate; 30:1) for 4 hr, LDH levels of culture supernatant was estimated. Significant suppression of LDH release by G-CSF in MTLC assay was observed.

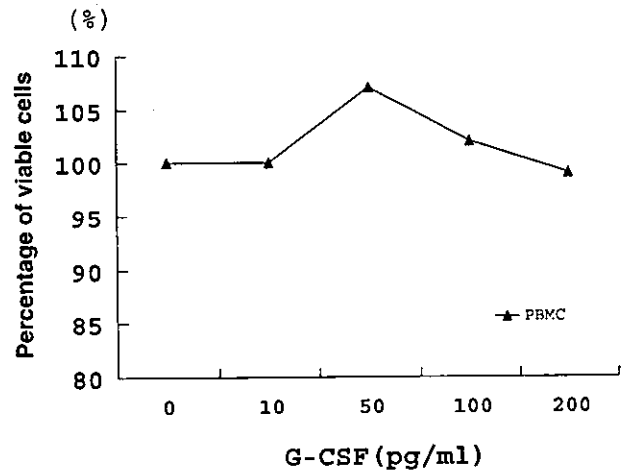

Fig. 6. Effects of granulocyte colony-stimulating factor (G-CSF) on viability of peripheral lymphocytes. In order to analyze the possible direct cytotoxicity of G-CSF on peripheral blood lymphocytes, we cultured unsorted peripheral mononuclear cells for 48 hr in the presence of various concentrations of G-CSF. Cell viability was measured by Trypan blue dye exclusion assay. G-CSF did not affect peripheral lymphocyte viability in the concentration range from 10 to 200 pg/mL.

disease-free interval as well as total survival of patient indicates that ATK activity is a meaningful prognostic indicator. This also provides a evidence for immunologic regulation of tumor growth and metastasis.[22] Antitumor immune response *in vivo* is the sum of CTLs activities, NK cell activities and other innate immune responses. Almost all components of the antitumor cellular immune response can be up-regulated by IL-2 and/or IL-12. G-CSF possibly acts as a general

immune suppressor for specific and innate immune reactions, because G-CSF receptor is expressed ubiquitously by $CD4^+$ or $CD8^+$ T cells and $CD16^+$ NK cells.

However, Lovgren et al. recently reported that the combination of Il-2, IL-15 and G-CSF enhanced cytotoxicity of cord BMC against breast carcinoma cell lines. They hypothesized that the increased cytotoxicity was mediated through (i) increase in CD56-positive cytotoxic cells; (ii) cytokine/cytotoxic factors produced by the effector cells, such as IFN-γ and perforin; (iii) stimulation by accessory cells, such as the dendritic cells.[24] It is noteworthy that their experiments were performed using healthy term cord BMC and established cell lines. Possibly their experimental model reflects alloimmune responses rather than antitumor-associated antigen-specific responses. Obviously our experimental model employing ATK reaction is more appropriate to mimic host-tumor immune reactions *in vivo*. We examined IFN-γ production in antigenically separated PBMCs because IFN-γ is a common cytokine produced both by T-cell lineage and NK cells. Production of IFN-γ does not require antigenic recognition.

We observed G-CSF-induced suppression of IFN-γ production of IL-12/IL-2-activated $CD4^+$, $CD8^+$, $CD16^+$ cells, which represent Th cells, suppressor/cytotoxic T cells and NK cells respectively. Many past reports indicated a similar Th2 directed immune responses in cancer-bearing patients.[25–27] We observed reduced IFN-γ production in the PBMC collected from the patients with ovarian carcinoma (Figs 2 and 3). These results coincide well with previous reports. Of interest, IFN-γ production is strongly suppressed by pharmacologic dosages of G-CSF in PBMCs collected from normal subjects as well as cancer-bearing patients. Hence, it can be said that G-CSF is a potent immune suppressor even for healthy persons.

The mechanism of this G-CSF-mediated suppression is so far unclear. Rutella et al. reported on the induction of T-regulatory 1 (Tr1) cells by G-CSF *in vivo*,[28] which does not appear to be plausible from our point of review.

We offer that G-CSF directly suppresses proinflammatory cytokine-induced activation in T and NK cells besides induction of immunosuppressive cytokines such as transforming growth factor (TGF)-β and IL-10.

Our preliminary results suggest involvement of suppressor cytokine signaling (SOCS) gene system in G-CSF-induced immune suppression (unpublished data).

In summary, stimulation of the peripheral blood $CD4^+$, $CD8^+$ T cells and $CD16^+$ NK cells by IL-2- and/or IL-12-induced IFN-γ production. IL-2/IL-12 also induced enhanced ATK activities by PBMCs against autologous ovarian carcinoma cells. On the contrary, G-CSF suppressed these activities in a dose-dependent manner.

These findings suggest a suppressive role of G-CSF in cancer immunology, in addition to its well-known tropic effects on hematopoiesis. At present we are analyzing the intracellular signal transduction system associated with human immune response. As clinicians, we have to avoid overuse of G-CSF in cancer patients, because it suppresses cellular immune function of cancer-bearing host.

Acknowledgments

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Mediation of β-Endorphin by the Isoflavone Puerarin to Lower Plasma Glucose in Streptozotocin-Induced Diabetic Rats

Abstract

We investigate the mechanism(s) of plasma glucose lowering action of puerarin in streptozotocin-induced diabetic rats (STZ-diabetic rats). Puerarin at the effective dosage to lower higher plasma glucose increased plasma β-endorphin-like immunoreactivity (BER) in STZ-diabetic rats. Both effects of puerarin were abolished by the pretreatment with prazosin. Also, puerarin enhanced BER release from isolated rat adrenal medulla in a concentration-dependent manner that can be abolished by prazosin. Moreover, bilateral adrenalectomy in STZ-diabetic rats eliminated the actions of puerarin including the plasma glucose lowering effect and plasma BER elevating effect. In addition, naloxone and naloxonazine inhibited the plasma glucose lowering action of puerarin. Unlike in wild-type diabetic mice, puerarin failed to lower the plasma glucose in opioid μ-receptor knockout diabetic mice. In conclusion, our results suggest that puerarin may activate α_1-adrenoceptors on the adrenal gland to enhance the secretion of β-endorphin to result in a decrease of plasma glucose in STZ-diabetic rats.

Key words

Puerarin · streptozotocin-induced diabetic rats · β-endorphin · α_1-adrenoceptors · opioid μ-receptors

Introduction

Diabetes, which ranks highly among the top ten causes of mortality throughout the world often leads to disability from vascular complications and limb amputation in addition to neurological complications and premature death [1]. With the rapid advancements in medicine, novel treatments with fewer side effects became feasible for the long-term control of this disorder. Of the herbal medications used in diabetic disorders, Puerariae Radix is mentioned as one of the useful crude materials in Oriental medicine [2]. Some isoflavones, such as: puerarin (a C-glucoside; Fig. 1), daidzein and daidzin, have been isolated as the main constituents of *Pueraria lobata* (Leguminosae) [3]. Actually, puerarin has been mentioned to decrease the serum cholesterol and higher glucose levels in addition to increasing coronary artery blood flow [4], [5], [6]. These observations suggest that puerarin seems to be helpful for diabetic treatment.

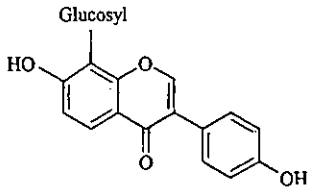

Fig. 1 The chemical structure of puerarin.

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Activation of opioid μ-receptors by either exogenous β-endorphin or chemical agents might improve the glucose homeostasis in diabetic rats under the absence of insulin [7], [8], [9]. Also, activation of α_1-adrenoceptors in adrenal medulla enhanced the secretion of β-endorphin from the rat adrenal gland [10]. It seems that activation of α_1-adrenoceptors or opioid μ-receptors may lower plasma glucose in rats with type I-like diabetes. Puerarin has the ability to activate α_1-adrenoceptors in cultured myoblast C_2C_{12} cells of mice [11]. However, the role of β-endorphin in the plasma glucose lowering action of puerarin is still unclear. Thus, we employed streptozotocin-induced diabetic rats (STZ-diabetic rats), an animal model for type 1-like diabetic mellitus, to investigate the action mechanism(s) of puerarin in relation to the secretion of β-endorphin-like immunoreactivity (BER) *in vivo*.

Materials and Methods

Materials

Puerarin (purity > 98%) was supplied by Professor Juntian Zhang (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China). Streptozotocin was obtained from Sigma Chemical Co. (St. Louis, USA). Naloxone, and naloxonazine were purchased from Research Biochemical Inc. (Natick, MA). A commercial kit for BER was purchased from Penisula Lab. Inc. (CA, USA).

Animals

Male Wistar rats aged 8 – 10 weeks, weighing 200 – 250 g were obtained from the Animal Center of National Cheng Kung University Medical College. Male wild-type (BDF1) mice and opioid μ-receptor knockout mice [12], aged 8 – 10 weeks, were obtained from Professor H. H. Loh (Minneapolis, USA). STZ-diabetic rats were prepared by giving an intravenous (*i.v.*) injection of STZ (60.0 mg/kg) into male Wistar rats. Mice with or without opioid μ-receptors also received an intraperitoneal injection (*i.p.*) of STZ at 50.0 mg/kg to induce diabetes according to the previous method [13]. Animals with plasma glucose concentrations of 20.0 mmol/L or greater in addition to polyuria and other diabetic features were considered as type 1-diabetes. Meanwhile, the plasma insulin level in STZ-diabetic rats became 1.24 ± 0.7 pmol/L (N = 8) in a way markedly lower than that of the normal rats (174.1 ± 5.6 pmol/L; N = 8) showing the insulin-dependent diabetes mellitus. All studies were carried out 2 weeks after the injection of STZ. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

Effect of puerarin on plasma β-endorphin-like immunoreactivity (BER) in STZ-diabetic rats

After fasting overnight, STZ-diabetic rats received an *i.v.* injection of puerarin at the desired doses and blood samples (0.1 mL) were collected under sodium pentobarbital anesthesia (30.0 mg/kg, *i.p.*) from the tail vein for measurement of plasma glucose and plasma BER. Concentration of plasma glucose was measured by the glucose oxidase method using an analyzer (Quik-Lab, Ames, Miles Inc., Elkhart, Indiana 46515, U.S.A.). The effective plasma glucose lowering action of puerarin at 15.0 mg/kg was found to produce in STZ-diabetic rats 30 min after an *i.v.* injection [6], the effect of puerarin on plasma BER was thus determined using blood samples collected at 30 min after single *i.v.* injection. STZ-diabetic rats that received a similar injection of the same volume of vehicle (distilled water containing 0.9% NaCl) used to dissolve puerarin were used as controls. Further experiments were performed with the pharmacological inhibitors, such as prazosin, naloxone or naloxonazine. These inhibitors were injected intravenously into fasting rats 30 min before the injection of puerarin. The BER in sample was determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Penisula Lab. Inc., CA, USA).

Effect of puerarin on plasma glucose concentrations in opioid μ-receptor knockout diabetic mice

The fasting STZ-diabetic mice with or without opioid μ-receptors were given an *i.v.* injection of puerarin at 15.0 mg/kg, the effective dose to lower plasma glucose in STZ-diabetic rats. After 30 min, blood samples (0.1 mL) were then collected from the blood vessel in the lower eye-lid of mice under anesthesia with pentobarbital (30.0 mg/kg, *i.p.*) with a chilled syringe containing 10 IU of heparin.

Isolation and incubation of adrenal medulla

The adrenal glands were quickly removed from the sacrificed STZ-diabetic rats and medullae were immediately dissected after the removal of cortex as described previously [14]. Tissues were cut in slices about 1 mm thick and then transferred into a glass tube with a mesh of nylon fitted at the bottom to permit free interchange with medium. Tissues were placed in a incubator for 15 min at 37 °C bubbled with a 95% O_2 and 5% CO_2 mixture under continued shaking with 2 mL of modified Kreb's solution (MKS, pH 7.4) [10]. Tissues were transferred to the fresh incubation tubes with or without prazosin at the indicated concentrations for 15 min at 37 °C [10]. The tissues were then incubated with puerarin at indicated concentrations under 37 °C for 30 min with continuous shaking at 40 cycles/min. Incubation was terminated by placing the tubes on the ice. The medium from each incubation was then collected and frozen at –70 °C until the assay of β-endorphin.

Adrenalectomized rats

Bilateral adrenalectomy was performed according to the previous study [15]. The sham-operated Wistar rats were served as controls [15]. Animals were allowed to recover for 2 weeks after the operation. The animals appeared alert and in good health. Then, they had diabetes induced by an injection of STZ as described above. The effect of puerarin at 15.0 mg/kg was determined using blood samples collected at 30 min after the single *i.v.* injection as described above.

Statistical analysis

Parametric data were expressed as the mean ± s.e.. The N in the text refers to the number of separate experiments. Repeated measures analysis of variance (ANOVA) was used to analyze the changes in plasma glucose and other parameters. Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate. The P value of 0.05 or less was considered as significant statistically.

Results and Discussion

Thirty minutes after treatment, puerarin increased the plasma BER level in STZ-diabetic rats from the basal level of 42.4 ± 4.8 pg/mL to

68.2 ± 7.5 pg/mL, 80.5 ± 6.9 pg/mL and 89.6 ± 8.1 pg/mL after dosing with 8.0 mg/kg, 12.0 mg/kg and 15.0 mg/kg, respectively (N = 8). Meanwhile, the plasma glucose concentration was decreased from 25.7 ± 2.8 mmol/L to 17.1 ± 3.1 mmol/L in rats receiving an *i.v.* injection of puerarin (15.0 mg/kg) (N = 8); the plasma glucose lowering activity of puerarin was about 33.8 ± 3.7%, which was similar to the previous report [6]. Thus, an elevation of β-endorphin seems to be related to the plasma glucose lowering action of puerarin in the absence of insulin. In fact, regardless of insulin regulation, physical exercise is considered beneficial in the improvement of type-1 diabetes [16]. Otherwise, endogenous β-endorphin was involved in the glucose homeostasis under the insulin deficient state [9]. The role of endogenous β-endorphin in the insulin-independent plasma glucose lowering action of puerarin was then investigated in diabetic rats with insulin deficiency.

In addition to the pituitary gland, the adrenal gland is known to be another source of β-endorphin [17]. An activation of α_1-adrenoceptors on the adrenal medulla to modify endogenous β-endorphin secretion has been recently documented [10]. Puerarin has an ability to activate α_1-adrenoceptors [11]. Thus, prazosin was employed to clarify the relationship between α_1-adrenoceptors and β-endorphin in the action of puerarin. As shown in Table **1**, the increase of plasma BER content induced by puerarin in STZ-diabetic rats was attenuated in a dose-dependent manner by prazosin given 30 min before the *i.v.* injection of puerarin (15.0 mg/kg). Similar to the effect on plasma BER level, the plasma glucose lowering action of puerarin (15.0 mg/kg) was reduced in a dose-dependent manner by the pretreatment with prazosin while prazosin (1.0 mg/kg) completely reversed these actions of puerarin. Moreover, prazosin (1.0 mg/kg) did not influence the basal plasma BER level or plasma glucose concentration in STZ-diabetic rats. Therefore, the increase of endogenous β-endorphin secretion via activation of α_1-adrenoceptors seems related to the plasma glucose lowering action of puerarin under the insulin deficient state.

In attempt to make sure of the role of adrenal gland, we employed the isolated adrenal medulla to investigate the direct effect of puerarin on the secretion of β-endorphin using an ELISA assay. We found that puerarin enhanced BER secretion from isolated adrenal medulla of STZ-diabetic rats from a basal level of 71.9 ± 6.5 pg/mg protein to 134.8 ± 10.3 pg/mg protein, 163.2 ± 9.8 pg/mg protein and 179.5 ± 11.5 pg/mg protein after dosing with 0.1 μmol/L, 1.0 μmol/L and 10.0 μmol/L, respectively (N = 8). Furthermore, prazosin at the effective concentration of 1.0 μmol/L reversed the puerarin (10.0 μmol/L)-stimulated BER secretion to 78.4 ± 9.5 pg/mg protein, that was near the basal level. Thus, activation of α_1-adrenoceptors in the adrenal medulla by puerarin to enhance β-endorphin secretion can be considered as the plausible mechanism.

Moreover, adrenalectomy was also carried out in STZ-diabetic rats. Two weeks after adrenalectomy, neither the basal plasma glucose nor the basal plasma BER was significantly different between adrenalectomized STZ-diabetic rats and the sham-operated group (Table **2**). However, both the decrease of plasma glucose and the increase of plasma BER in response to puerarin were not seen in STZ-diabetic rats with bilateral adrenalectomy, while these effects persisted in the sham-operated STZ-diabetic rats (Table **2**). Thus, secretion of endogenous β-endorphin from the adrenal gland seems to be involved in the action of puerarin.

It has been indicated that the opioid μ-receptor participates in the regulation of glucose metabolism during an insulin deficient state [7], [8], [9]. Endogenous β-endorphin has physiological actions that are mediated by the opioid μ-receptor [18], [19]. Thus, blockers of the opioid μ-receptor were employed to investigate the action of puerarin in STZ-diabetic rats. The dose-dependent inhibitory effects of naloxone and naloxonazine on the action of puerarin in STZ-diabetic rats are shown in. In the presence of 10.0 μg/kg naloxone, the plasma glucose concentration in STZ-diabetic rats treated with 15.0 mg/kg puerarin reversed to a value near the basal level. Similar results were obtained in the naloxonazine (10.0 μg/kg)-pretreated group. Moreover, both naloxne and naloxonazine did not modify the basal plasma glucose level of STZ-diabetic rats even at the highest dose (Table **3**). This suggests the involvement of opioid μ-receptors in the plasma glucose lowering action of puerarin in STZ-diabetic rats. However, multiple influences of these antagonists in addition to the blockade of opioid μ-receptors should be concerned. Thus, we then employed opioid μ-receptors knockout mice to confirm the role of opioid μ-receptors

Table **1** Effects of prazosin on the puerarin-induced changes of β-endorphin-like immunoreactivity (BER) and glucose concentrations in plasma of STZ-diabetic rats[a]

	Plasma glucose (mmol/L)	*Plasma BER (pg/mL)*
Basal	25.8 ± 3.2	44.3 ± 4.6
Puerarin (15.0 mg/kg, *i.v.*)		
+ Vehicle	17.2 ± 2.9*,*	92.3 ± 6.1**
+ Prazosin (mg/kg, *i.v.*)		
0.1	19.2 ± 2.8*	76.4 ± 5.3**
0.5	22.1 ± 3.5	60.7 ± 6.4*
1.0	25.1 ± 3.3	45.5 ± 5.8
Prazosin (1.0 mg/kg, *i.v.*)	26.1 ± 3.9	42.5 ± 6.9

[a] Values (mean ± s.e.) were obtained from each group of 8 animals. Basal level shows the value from fasted animals treated with vehicle (distilled water containing 0.9% NaCl) used to dissolve the test reagents at the same volume.

* $P < 0.05$ and ** $P < 0.01$ compared to basal value, respectively.

Table **2** Effect of adrenalectomy on the puerarin-induced changes of β-endorphin-like immunoreactivity (BER) and plasma glucose concentration in STZ-diabetic rats[a]

	Adrenalectomized Group	*Sham-Operated Group*
Plasma glucose (mmol/L)		
Basal	25.9 ± 3.7	25.4 ± 4.6
Vehicle	25.5 ± 5.1	25.2 ± 4.3
Puerarin (15.0 mg/kg, i.v.)	25.1 ± 3.9	16.9 ± 3.5**
Plasma BER (pg/mL)		
Basal	43.2 ± 4.7	44.6 ± 5.1
Vehicle	42.6 ± 5.3	45.2 ± 4.3
Puerarin (15.0 mg/kg, i.v.)	46.1 ± 4.9	94.3 ± 3.6**

[a] Values (mean ± s.e.) were obtained from each group of 8 animals. Basal level shows the value from fasted animal without treatment. The vehicle of distilled water containing 0.9% NaCl used to dissolve puerarin was given at the same volume.

** $P < 0.01$ compared to basal value in each group.

Table 3 Effects of opioid μ-receptors antagonists on the puerarin-induced lowering of plasma glucose in STZ-diabetic rats[a]

	Plasma glucose (mmol/L)
Basal	25.2 ± 4.2
Puerarin (15.0 mg/kg, *i.v.*)	
+ Vehicle	17.1 ± 3.1**
+ Naloxone (μg/kg, *i.v.*)	
1.0	19.1 ± 3.4
5.0	22.4 ± 2.5
10.0	24.9 ± 4.3
+ Naloxonazine (μg/kg, *i.v.*)	
1.0	20.3 ± 2.2
5.0	23.6 ± 3.5
10.0	25.8 ± 3.1
Naloxone (10.0 μg/kg, *i.v.*)	25.7 ± 4.3
Naloxonazine (10.0 μg/kg, *i.v.*)	25.4 ± 3.7

[a] Values (mean ± s.e.) were obtained from each group of 7 animals. Basal level shows the value from fasted animals treated with vehicle (distilled water containing 0.9% NaCl) used to dissolve the test reagents at the same volume.

** $P < 0.01$ compared to basal value, respectively.

activation in the action of puerarin. Fig. 2 shows that the plasma glucose concentrations of opioid μ-receptors knockout diabetic mice were not significantly changed by puerarin at 15.0 mg/kg. However, in the presence of opioid μ-receptors in diabetic mice, puerarin (15.0 mg/kg) decreased the plasma glucose concentration from 25.1 ± 2.8 mmol/L to 16.9 ± 2.2 mmol/L. The plasma glucose lowering activity of puerarin (15.0 mg/kg) in these wild-type diabetic mice was about 32.6 ± 3.7%, which was near to that (33.8 ± 3.2%) produced in STZ-diabetic rats. These data support the essential role of opioid μ-receptors in plasma glucose lowering action of puerarin under insulin deficient state.

In conclusion, our results suggest that an activation of α_1-adrenoceptors in adrenal medulla by puerarin may enhance the secretion of endogenous β-endorphin from the adrenal gland of STZ-diabetic rats. Thus, the improvement of glucose utilization by puerarin to lower the plasma glucose concentration in STZ-diabetic rats is mainly mediated by endogenous β-endorphin.

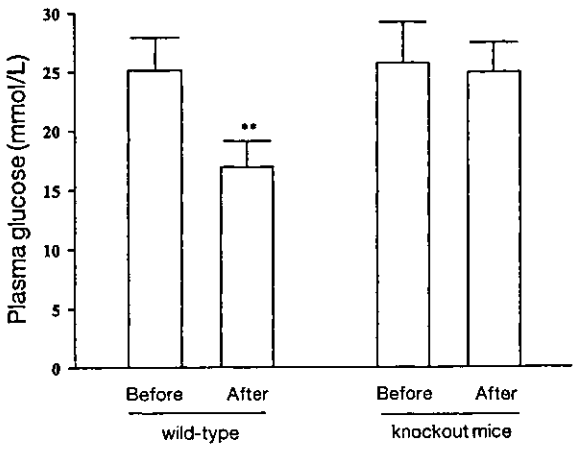

Fig. 2 Effects of puerarin on plasma glucose concentrations in opioid μ-receptors knockout mice and wild-type control. Values of mean and s.e. bar were obtained from each group of 6 animals. ** $P < 0.01$ compared to data obtained from animals before treatment in each group.

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The plasma glucose lowering action of tetrandrine in streptozotocin-induced diabetic rats

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Abstract

The effect of tetrandrine, an active principle of *Stephaniae tetrandrae*, on the plasma glucose level in streptozotocin-induced diabetic rats (STZ-diabetic rats) was investigated. A single intravenous injection of tetrandrine decreased the plasma glucose in a dose-dependent manner in STZ-diabetic rats. Moreover, tetrandrine (1.0 mg kg^{-1}) significantly attenuated the rise in plasma glucose induced by the intravenous glucose challenge test in normal rats. A stimulatory effect of tetrandrine on glucose uptake was obtained in soleus muscles isolated from STZ-diabetic rats with a concentration-dependent manner from 0.01 to 10.0 μmol L^{-1}. The increase in glucose utilization by tetrandrine was further characterized using the enhancement of glycogen synthesis in the hepatocytes of STZ-diabetic rats. These results suggest that tetrandrine has the ability to enhance glucose utilization in peripheral tissue, resulting in the lowering of plasma glucose in diabetic rats lacking insulin.

Introduction

Diabetes, which ranks highly among the top ten causes of mortality around the world, often leads to disability from the vascular complications of coronary artery disease, cerebrovascular disease, renal failure, blindness and limb amputation in addition to neurological complications and premature death (Lopez-Candales 2001). With recent rapid advancements in medicine, novel treatments with fewer side-effects have became feasible for the control of this disorder.

Tetrandrine (6,6′,7,12-tetramethoxy-2,2′-dimethylberbaman; Figure 1) is a bisbenzyl tetrahydroisoquinoline alkaloid extracted from a herb, the dry root of *Stephaniae tetrandrae* S. Moore (Menispermaceae), which has been widely used as an analgesic or anti-hypertensive drug in Oriental countries (Sutter & Wang 1993). The main ingredients in this herb are tetrandrine and fangchinoline (Sutter & Wang 1993). Tetrandrine works as a calcium entry blocker (Felix et al 1992), showing various actions, such as the modulation of cardiovascular disorders (Huang & Hong 1998), and anti-tumour (Lee et al 2002) and anti-inflammatory effects (Shen et al 1999). Fangchinoline is thought to be less potent than tetrandrine as a vasodilator or calcium channel blocker (Kim et al 1997). Tetrandrine has also been documented to have antioxidant properties (Cao 1996). Moreover, tetrandrine has been reported to prevent the development of spontaneous diabetes in BioBreeding rats (Lieberman et al 1992) and to protect pancreatic islet beta cells from the injuries caused by alloxan (Sun et al 1994). These results indicate that tetrandrine may be helpful in the prevention and/or handling of diabetes. However, the direct effect of tetrandrine on glucose metabolism is still unclear. Thus, we investigated the effect of tetrandrine on plasma glucose in diabetic rats lacking insulin.

Materials and Methods

Materials

Streptozotocin, tetrandrine and cytochalasin B were purchased from Sigma-Aldrich, Inc. (St Louis, MO) and 2-[1-^{14}C]-deoxy-D-glucose ([^{14}C]-2-DG) and [U-^{14}C]-glucose

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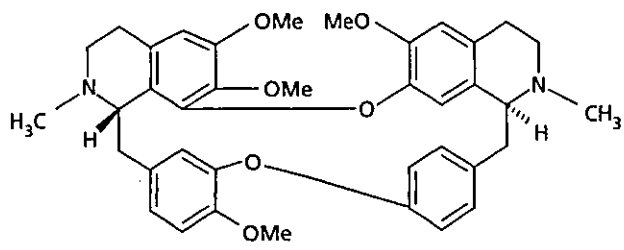

Figure 1 Chemical structure of tetrandrine.

were obtained from NEN Research (Boston, USA). Bovine insulin was obtained from Novo Nordisk (Bagsvaerd, Denmark). Protein assay kit was the product of BioRad (Richmond, CA). All other standard reagents were purchased from Sigma-Aldrich, Inc. (St Louis, MO).

Animals

Male Wistar rats, weighing 200–250 g, were obtained from the Animal Center of the National Cheng Kung University Medical College. Streptozotocin-induced diabetic rats (STZ-diabetic rats) were prepared by intravenous (i.v.) injection of STZ (60.0 mg kg^{-1}) into male Wistar rats, 8–10 weeks of age. Animals were considered to be diabetic if they had plasma glucose concentrations of 20 mmol L^{-1} or greater in addition to polyuria and other diabetic features. Plasma insulin levels in STZ-diabetic rats were reduced to 1.33 ± 0.8 pmol L^{-1} ($n = 8$) following STZ injection, a level markedly lower than that of the normal rats (161.1 ± 3.4 pmol L^{-1}; $n = 8$), indicating insulin-dependent diabetes mellitus. All studies were carried out 2 weeks after the injection of STZ. All animal procedures were performed according to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

Effect of tetrandrine on plasma glucose concentrations in STZ-diabetic rats

After fasting overnight, STZ-diabetic rats received an i.v. injection of tetrandrine into the femoral vein at the desired doses. Under anaesthesia with sodium pentobarbital (30.0 mg kg^{-1}, i.p.), blood samples (0.1 mL) were collected from the tail vein for measurement of plasma glucose concentrations. In the preliminary experiments, tetrandrine at 1.0 mg kg^{-1} was found to produce the maximal plasma glucose lowering effect in STZ-diabetic rats 30 min after a single i.v. injection. Thus, the effects of tetrandrine on plasma glucose were determined using blood samples collected 30 min after the injection. Comparative studies of the change in mean arterial blood pressure were also carried out in STZ-diabetic rats treated with tetrandrine. STZ-diabetic rats that received a similar injection of the same volume of vehicle (distilled water containing 0.9% (w/v) sodium chloride) used to dissolve tetrandrine were used as controls.

Intravenous glucose challenge test

The basal plasma glucose level was obtained from normal Wistar rats under pentobarbital anaesthesia prior to the intravenous glucose challenge test (IVGCT). A solution of tetrandrine at 1.0 mg kg^{-1}, the effective dose for lowering plasma glucose in rats, or the same volume of vehicle was injected into the femoral vein of rats. Thirty minutes later, a glucose dose of 60.0 mg kg^{-1} was injected to induce IVGCT. Blood samples from the tail vein were also drawn at 5, 10, 20, 30, 60, 90 and 120 min following IVGCT for measurement of plasma glucose levels. The blood samples (0.1 mL) obtained immediately from the tail veins of these rats were regarded as 0 min samples. Rats were maintained under pentobarbital anaesthesia throughout the IVGCT.

Laboratory determinations

The concentration of plasma glucose was measured by the glucose oxidase method using an analyser (Quik-Lab, Ames, Miles Inc., Elkhart, IN). Mean arterial blood pressure was measured using a non-invasive tail-cuff monitor (UR-5000, Ueda Company, Tokyo, Japan) in conscious diabetic rats. Blood pressure was expressed as the mean of at least four measurements.

Measurement of glucose uptake into soleus muscle

Soleus muscle was isolated from STZ-diabetic rats and divided into long longitudinal strips (35–25 mg per strip) as described previously (Cheng et al 2001). After a 30-min pre-incubation period, the muscle tissue was transferred to fresh incubation flasks, then incubated with tetrandrine at the desired concentrations at 37 °C for 30 min under continuous shaking at 40 cycles min^{-1}. The muscle tissue was subsequently incubated with 50 μL Krebs–Ringer bicarbonate buffer (KRBB) containing [^{14}C]-2-DG (1 μCi mL^{-1}) for 5 min in the presence of tetrandrine at 37 °C. Reactions were terminated by quickly blotting the muscles and dissolving them in 0.5 mL of 0.5 M NaOH for 45 min before neutralization with 0.5 mL 0.5 M HCl. After centrifugation, 800 μL of each supernatant was mixed with 1 mL of aqueous counting scintillant and the radioactivity was determined using a β-counter (Beckman LS6000). Uptake of [^{14}C]-2-DG, assessed after pre-incubation of the muscle with 20 μmol L^{-1} cytochalasin B, was subtracted from the total muscle-associated radioactivity. Specific [^{14}C]-2-DG uptake was expressed as pmol in 5 min or as the percentage of basal level that was obtained from sample incubated with KRBB only.

Measurement of glycogen synthesis in hepatocytes

The measurement of glycogen synthesis in hepatocytes isolated from STZ-diabetic rats was carried out as described previously (Cheng et al 2001). After the 30 min pre-incubation period in KRBB at 37 °C, 2×10^6 hepatocytes were transferred to fresh incubation flasks containing