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Suppression of human lymphocyte proliferation and cytotoxic T lymphocyte generation by a soluble factor derived from K562 cells

K. Hayamizu, H. Yahata, Y. Fukuda, and K. Dohi

The Second Department of Surgery, Hiroshima University School of Medicine, Hiroshima, Japan

Abstract. The human myeloid leukemia cell line, K562, secrets a lymphocyte growth-suppressive factor (LGSF). We report our investigation of the immunological and chemical features of this factor. LGSF showed no cytolytic effect on human peripheral blood mononuclear cells (PBMC). Nevertheless, LGSF adequately suppressed in vitro alloantigen- or mitogen (PHA, ConA, PWM)-stimulated human PBMC proliferation and alloantigen-induced cytotoxic T lymphocyte generation, in antigen-nonspecific and LGSF concentration-related manners. LGSF retained activity after filtration through a 0.22 μm filter membrane and storage at −80 °C for 6 months. Ultrafiltration experiments indicated that suppressive activity was retained in the higher molecular weight fraction (MW > 100000) and that the activity was heat-labile at 56 °C for 30 min. These results strongly suggested that LGSF is a noncytotoxically immunosuppressive substance with a high molecular weight.

Key words: Immunosuppression – Cytokine – Human leukemia cell line – Lymphocyte growth-suppressive factor – Cytotoxic T lymphocyte

The continuous cell line K562 was originally established by Lozzio et al. in 1975 from the pleural effusion of a patient with chronic myeloid leukemia [4]. The K562 cells are so sensitive to the direct tumoricidal activity of NK cells, K cells, and macrophages that they have been used as the standard target cells in such assays [3]. Some investigators have shown that K562 cells are able to diminish immunological reactions in vitro [1, 2, 5]. Olofsson et al. have reported that K562 cells produce a potent inhibitor of PHA-activated T-cell proliferation [5]. We confirmed the existence of lymphocyte growth-suppressive factor (LGSF) in the supernatant of cultured K562 cells which had been subcultured in our laboratory. In the present study we investigated further the immunosuppressive activities of the factor against mitogen-stimulated lymphocyte growth, mixed lymphocyte reactions, and cytotoxic T lymphocyte generation.

Offprint requests to: Keisuke Hayamizu M.D., 1-2-3, Kasumi, Minami-ku, Hiroshima, 734, Japan

Materials and methods

Cell culture. K562 cells, a gift from the Japanese Cancer Research Resources Bank, were maintained in RPMI 1640 (Gibco, Grand Island, N.Y.) containing 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), supplemented with 10% fetal calf serum [(FCS) Gibco] in 25 mm^2 tissue culture flasks (3013; Falcon, Oxnard, Calif.) at 37 °C in 5% CO_2 humidified atmosphere. They were maintained thus in our laboratory for more than 2 years.

Preparation of LGSF. For the production of effective LGSF, exponentially growing K562 cells were cultured at 5×10^5 cells/ml in RPMI 1640, supplemented with 10% heat-inactivated pooled human AB serum [complete medium (CM)] or without serum for 2 days, then centrifuged at 3000 rpm for 20 min. The supernatant harvested was passed through a 0.22 μm filter (Sterivex-GV; Millipore, Bedford, Mass.) and stored in conical tubes (2097; Falcon) at −80 °C until used in the suppressor assays described below. Concentrations of LGSF presented in this paper are the final volume/volume ratio or its percentage expressions.

Preparation of lymphocytes. Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood from healthy donors by using standard Ficoll-Hypaque density gradient centrifugation (Lymphoprep 1077; Nycomed, Oslo, Norway).

Mitogen-stimulated PBMC growth. Human PBMC (2×10^5) were cultured with serial dilutions of LGSF in 200 μl of CM, supplemented with 0.1% PHA-P (Difco, Detroit, Mich.) or 64 μg/ml ConA (Miles Yedaltd) for 3 days, or with 1% PWM (Gibco) for 5 days respectively, in each well of a flat-bottom 96-well microtiter plate (3072; Falcon). Tritiated thymidine (^{3}H-TdR; Amersham, Buckinghamshire, England) (0.5 μCi/well) was added for the last 12 h of the culture period. The cells were subsequently harvested and assayed for ^{3}H-TdR uptake. The resultant suppression of the response is expressed by the percentage suppression calculated according to the formula:

% Suppression = (1 − cpm of LGSF-treated cultures/cpm of control cultures) × 100

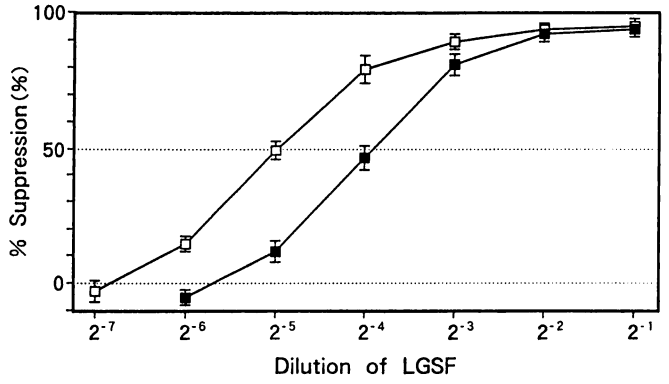

Fig. 1. Titration of suppressive activity of LGSF against PHA-stimulated lymphocyte growth. PBMC (2×10^5/culture) were cultured with 0.1% PHA and serial dilutions of LGSF prepared from the supernatant of K562 cell cultures with (–□–) or without (–■–) 10% pooled human AB serum in triplicate for 3 days. Cultures were assayed for ^{3}H-TdR uptake and the percentage suppression was calculated. ^{3}H-TdR uptake by the culture containing no LGSF was 69730 ± 1642 cpm. Data presented are the mean ± SD

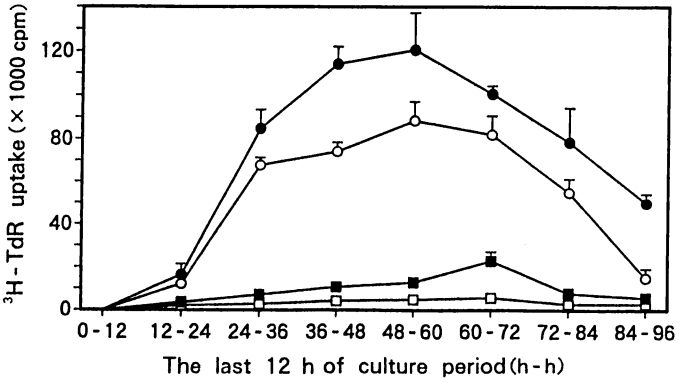

Fig. 2. Effect of LGSF on the kinetics of PHA-stimulated lymphocyte growth. PBMC (2×10^5/culture) were cultured with 2^{-1} (50%, –□–), 2^{-3} (12.5%, –■–), 2^{-5} (3.125%, –○–) LGSF or without it (0%, –●–), in the presence of 0.1% PHA. Three cultures were harvested every 12 h, pulsed with 0.5 μCi ^{3}H-TdR for the last 12 h of each culture period. Subsequently, the cultures were assayed for ^{3}H-TdR uptake. Data presented are the mean ± SD of the velocity of ^{3}H-TdR uptake (cpm/12 h)

The LGSF titers were expressed as the reciprocal dilutions causing 50% suppression.

One-way mixed lymphocyte reaction (MLR). Responder PBMC (1×10^5) and 2000 rad-irradiated stimulator allogeneic or autologous PBMC (1×10^5) were cultured together with 25% LGSF in 200 μl of CM in each well of the microtiter plate for 6 days. ^{3}H-TdR was added for the last 18 h of the culture period. The percentage suppression was calculated as described above.

^{51}Cr release assay. For the generation of cytotoxic T lymphocytes (CTL), PBMC (1×10^6/ml) were cultured with 2000 rad-irradiated PBMC (1×10^6/ml) and various dilutions of LGSF in a culture tube for 5 days. For the CTL assay, 3-day PHA-pretreated and ^{51}Cr ($Na_2{}^{51}CrO_4$; New England Nuclear, Boston, Mass.)-labeled target PBMC (1×10^4) and effector cells (5×10^5, 2.5×10^5, 1.25×10^5) in 200 μl of CM were put into each well of a U-bottom microtiter plate (Nunclon, Denmark). For the direct LGSF cytotoxicity assays, 50% LGSF was used instead of the effector cells. After 4 h of incubation at 37 °C, 100 μl of supernatant was collected and counted in a gamma counter. The percentage cytotoxicity was calculated using the following equation:

% Cytotoxicity = (experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(maximum ^{51}Cr release – spontaneous ^{51}Cr release) × 100

Ultrafiltration experiment. LGSF was filtrated under high pressure using a Molcut-L (Millipore), incorporating the 100000 nominal molecular weight limit (NMWL) membrane, and was separated into 2 fractions: the 10-fold-condensed fraction and the membrane breakthrough fraction with a lower molecular weight than 100000 daltons. Subsequently each fraction was tested for PHA-stimulated PBMC growth-suppression assay.

Results

Effect of LGSF on mitogen-induced lymphocyte growth (Table 1). LGSF at a concentration of 25% (volume/volume) inhibited significantly the lymphocyte growth activated by the mitogens PHA, ConA and PWM. The mean percentage suppression for the 3 donors was 88% ± 3%, 89% ± 3% and 92% ± 2% respectively.

Titration of LGSF (Fig. 1). In the PHA-stimulated lymphocyte growth-suppression assay, the dose-response curve for LGSF was sigmoid shaped. The percentage suppression ranged between –5% and 95%, over a 32-fold dilution of LGSF. The presence of serum did not affect the shape of the curve, but the LGSF titer required to produce the same effect without serum was 2.1-fold lower than that with 10% human serum.

Effect of LGSF on the kinetics of PHA-stimulated lymphocyte growth (Fig. 2). LGSF suppressed ^{3}H-TdR uptake throughout the entire culture period. The peak of ^{3}H-TdR uptake under various LGSF dilutions decreased in a LGSF dose-dependent way, but the culture time corresponding to peak uptake was almost constant.

Effect of LGSF on one-way MLR (Table 2). The 25% LGSF suppressed the proliferative response of lymphocytes stimulated by alloantigen. In six stimulator-respon-

Table 1. Effect of LGSF on mitogen-stimulated lymphocyte growth

Human PBMC	^{3}H-TdR uptake (cpm) Mitogen		
	PHA	ConA	PWM
KH	5369 ± 311[a] 69515 ± 1642 (92.3)[b]	4361 ± 109 29451 ± 1424 (85.2)	3383 ± 803 25329 ± 1629 (86.6)
HY	8092 ± 230 60613 ± 648 (86.7)	2774 ± 145 33840 ± 795 (91.8)	2437 ± 34 29523 ± 3056 (91.7)
HW	6313 ± 187 44086 ± 1335 (85.7)	5337 ± 910 48857 ± 3892 (89.1)	2782 ± 405 30668 ± 900 (90.9)

[a] Data presented are the mean cpm of triplicates ± SD. Upper data are ^{3}H-TdR uptake of the culture with 25% LGSF and lower data are that of control

[b] Values in brackets indicate percentage suppression

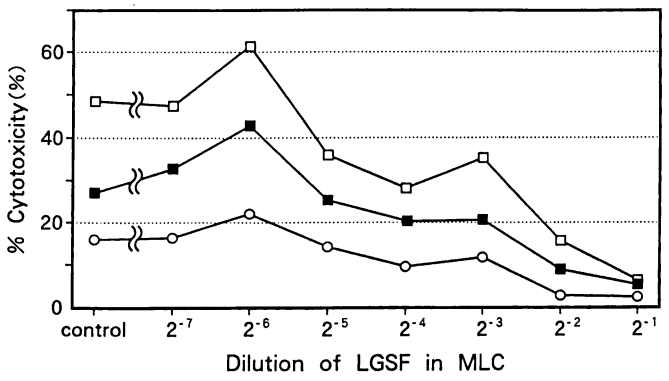

Fig. 3. Effects of LGSF concentration in the MLC on CTL generation. One-way MLCs were performed with serial dilutions of LGSF for 5 days and washed to remove the factor. Thereafter CTL activity of each MLC to stimulator (10^4/culture) as target was tested for ^{51}Cr release assay in duplicate. Effector/target cell ratios were 50/1 (– □ –), 25/1 (– ■ –) and 12.5/1 (– ○ –) respectively. Maximum ^{51}Cr released by lysed target cells per well was 5572 ± 73 cpm, and ^{51}Cr released spontaneously was 558 ± 11 cpm. The percentage cytotoxity of each test was calculated and the mean are presented

der allogeneic combinations among three donors (KH, MI, HW) expressing different HLA types, the mean percentage suppression was 85 % ± 4 %.

Effect of LGSF on alloantigen-induced CTL generation (Fig. 3). The addition of LGSF to MLC caused a decrease in the percentage cytotoxicity of the CTL generated against the allogeneic target PBMC. The percentage cytotoxicity was suppressed from 48 % to 7 % (E/T = 50), and the amount of decrease was dependent on the LGSF concentration in the MLC.

Direct cytotoxicity of LGSF. The percentage cytotoxicity of 50 % LGSF against PBMC in direct ^{51}Cr release assays was only 0.3 %. The cell viability for trypan blue exclusion of the 2-day PHA-stimulated cultures with or without 50 % LGSF was 95 % ± 11 % and 93 % ± 15 % respectively, and the difference was not significant using Student's *t*-test. Therefore, these data suggested that the inhibition occurs via a noncytotoxic mechanism.

Chemical features of LGSF. LGSF was stored at – 80 °C in a stable condition for more than 6 months. Ultrafiltration experiments showed a 5.3 times increase in the LGSF titer of a 10-fold condensed fraction of the initial filtered supernatant using a membrane with a 100000 dalton NMWL, and the fraction that passed through the membrane revealed no suppressive activity. The fraction with the higher titer completely lost activity after incubation at 56 °C for 30 min.

Table 2. Effect of LGSF on one-way mixed lymphocyte reaction

Responder	^{3}H-TdR uptake (cpm) Stimulator		
	KH	MI	HW
KH	1639 ± 532[a, NS] 470 ± 993 (– 248.7)[b]	7044 ± 437 29258 ± 3148 (75.9)	5027 ± 1005 31030 ± 4018 (83.8)
MI	4471 ± 838 32050 ± 1762 (86.0)	1152 ± 147[NS] 335 ± 283 (– 243.4)	3334 ± 415 26550 ± 876 (87.4)
HW	2607 ± 156 23119 ± 1557 (88.7)	2313 ± 304 18698 ± 2165 (87.6)	1460 ± 88[NS] 848 ± 609 (– 72.3)

[a] Data presented are the mean cpm of triplicates ± SD. Upper data are ^{3}H-TdR uptake of the culture with 25 % LGSF and lower data are that of control

[b] Values in brackets indicate percent suppression

[NS] The difference between the data of LGSF-containing cultures and control are not significant ($P > 0.05$) for Student's *t*-test. Other data are significant ($P < 0.01$)

Discussion

We investigated the possibility that established human hematopoietic cell lines produce active suppressive factors against normal human lymphocyte proliferation. We found that the supernatant of K562 cell cultures showed detectable suppressive activity, and called the factor LGSF. Lozzio et al. have determined in full the character of original K562 cells, and found them to produce neither immunoglobulins nor interferons, to be free of Epstein-Barr virus and herpes-like virus particles, and to show no reverse transcriptase activity [3]. Olofsson et al. have already reported that K562 cells produce an inhibitor of cell growth. The factor is restricted to activity against hematopoietic cells. It is most active against PHA-activated T cells and myeloid stem cells, less active against erythroid precursors, and does not inhibit fibroblasts or established lines of epithelioid cells or B cells [5]. These facts gave us hope for the possibility of a clinical application for LGSF as an immunosuppressive agent with few side effects.

In this study, further immunological features were examined, and LGSF produced by K562 cells was found to suppress mitogen-stimulated lymphocyte growth not only by PHA but also by ConA and PWM in a dose-dependent manner, and to inhibit alloantigen-stimulated lymphocyte growth and CTL generation alloantigen-nonspecifically, via a noncytotoxic mechanism.

Because of their high sensitivity K562 cells are used as the standard targets in NK assays, and also in macrophage tumoricidal assays [3]. The NK sensitivity of K562 cells is diminished by a differentiating agent, sodium butyrate [1] and the anti-K562 cytotoxicity of monocytes is inhibited by the supernatant of K562 cell cultures [2]. Although the precise mechanism of this sensitivity modulation is unknown, it is interesting that our subcultured K562 cells displayed potent lymphocyte growth-suppressive activity, despite retaining NK sensitivity.

As for the chemical features of LGSF, the suspision that LGSF was an altered serum protein was ruled out by the fact that K562 cells produced this factor in a serum-free medium. In addition, LGSF was secreted only during the log-growth phase of K562 cells, and filtered through 0.22 μm pores, so the possibility that LGSF represented the influence of contaminating organisms in the supernatant was also excluded.

This study provided evidence for the production of an efficient immunosuppressive substance with a high molecular weight by the leukemic cell line K562, which showed cytostatic effects on human lymphocytes. Studies on the application of condensed LGSF for the prevention of graft rejection in vivo in animals, and the biochemical purification of LGSF are in progress.

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