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In vivo Effect of Recombinant Human Leukemia Inhibitory Factor in Primates

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Leukemia inhibitory factor (LIF) is known to be a causative factor for cachexia and thrombocytosis in nude mice bearing human cancer cells. In the present study, we investigated whether recombinant human (rh) LIF can induce these biological activities in a primate model. rhLIF was synthesized by the expression of LIF protein in *Escherichia coli*. rhLIF (5, 20, or 80 μg/kg) was administered subcutaneously twice daily to cynomolgus monkeys for 14 consecutive days. A remarkable decrease of body weight (10%) was observed in the 80 μg/kg/day group. Approximately two-fold increases in platelet counts were observed at doses higher than 5 μg/kg/day when compared with control counts. These biological effects disappeared soon after the cessation of rhLIF treatment. Macroscopically, a remarkable reduction in subcutaneous fatty tissues and severe splenomegaly were observed. The results of this study demonstrate that rhLIF induces weight loss and thrombocytosis in a primate model.

Key words: Cachexia — Thrombopoiesis — LIF — Primate

Leukemia inhibitory factor (LIF) has pleiotropic biological effects on human leukemic cells, macrophages, osteoblasts, myoblasts, liver cells, megakaryocytes, preadipocytes and nerve cells.[1-14] Murine and human LIF cDNAs have been successfully cloned.[15,16]

We previously reported that LIF is a causative factor responsible for cancer cachexia syndrome in tumor-bearing nude mice.[17,18] We also purified LIF, based on monitoring of the growth-stimulating activity towards a megakaryoblastic cell line, CMK cells, from conditioned media of three cancer cell lines which exhibited thrombocytosis as well as cachexia in tumor-bearing nude mice. The latter observation raised the possibility that LIF could be a hematopoietic factor responsible for thrombocytosis in animal models. The present study was undertaken to elucidate whether rhLIF can induce cachexia and thrombocytosis in a primate model, cynomolgus monkeys.

MATERIALS AND METHODS

Animals Ten adult female cynomolgus monkeys weighing between 2.3 and 3.3 kg were housed individually in stainless steel cages in an air-conditioned room at 24±2°C with humidity between 50–60%.

Synthesis of recombinant human (rh) LIF Oligodeoxyribonucleotides encoding human LIF (1–180) were synthesized chemically with a model 394 DNA synthesizer (Applied Biosystems, Foster City, CA). The DNA fragments of 50–60 bases were ligated and inserted into an expression vector, pMAL-c plasmid (New England Biolabs, Beverly, MA), to express rhLIF (1–180) as a fusion protein with maltose-binding protein in *Escherichia coli* (*E. coli*). Transformed bacterial cells were grown in LB medium and induced to express the fusion protein. The crude extracts were applied to an amylose column, and the fusion protein was eluted with an excess of maltose. The desired fractions were collected and treated with the specific protease, factor Xa, to produce the mature rhLIF (1–180). The protein was further purified by two-step, reversed-phase, high-performance liquid chromatography (HPLC) on C18 (SynChropak RP-P, 10×250 mm, SynChrom, Lafayette, IN) and C8 (COSMOSIL 5C8-300, 4.6×100 mm, Nacalai Tesque, Kyoto) columns. The purity was over 95% as determined by densitometric analysis after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. The structure of the protein was confirmed by analysis of the *N*-terminal amino acid sequence. The amount of the recombinant protein was estimated by amino acid composition analysis, and the concentrations of samples were determined from the A210 peak areas on the final HPLC compared with that of a known amount of rhLIF (1–180). This preparation was used for administration to monkeys.

Administration of rhLIF to monkeys Specific activity of rhLIF was estimated as 2×10^7 U/mg protein by means of colony assay with CMK cells. One unit represents the same number of CMK colonies formed in a dish. The rhLIF solutions were prepared by reconstituting freeze-dried rhLIF with sterile phosphate-buffered saline (PBS) containing 1% heat-inactivated monkey serum. Five micrograms, 20 μg or 80 μg/kg/day rhLIF was adminis-

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tered subcutaneously twice daily from day 1 for 14 consecutive days. PBS containing 1% heat-inactivated monkey serum alone was used for the control group. The endotoxin in rhLIF solution was confirmed to be negative by means of the *Limulus* test.

Hematological studies Blood samples were obtained before treatment and on days 2, 4, 6, 10, 15, 20, 25, and 30 after the start of the injections. The parameters measured were the total counts of red blood cells (RBCs), white blood cells (WBCs), platelets, and the levels of hemoglobin and hematocrit (Sysmex 2000, Toa Medical Electronics, Kobe). Differential counts of WBCs were done with smear preparations stained with May-Giemsa. Bone marrow sections were obtained on day 15 from one of the monkeys receiving 80 μg/kg/day and from one of the control monkeys.

Blood chemical parameters and serum C-reactive protein (CRP) Total proteins, albumin, glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), lactate dehydrogenase (LDH), total cholesterol (T-Cho), triglyceride (TG), urea nitrogen (BUN), creatinine and calcium in sera were measured by means of a 706D automatic analyzer (Hitachi, Tokyo). Serum CRP was assayed by a nephelometric method using TIA test-CRP (Nissui Pharmaceutical, Tokyo).

Effect of rhLIF on body weight in monkeys During the experiments, each monkey in the control or rhLIF-injected groups was weighed daily.

Serum LIF levels in monkyes injected with rhLIF LIF concentrations in sera from each of the control and 5, 20, or 80 μg/kg/day rhLIF-injected monkeys were assayed using an enzyme-linked immunosorbent assay kit specific for human LIF ("Quantikine," R & D Systems, MN). Sera were obtained pre-treatment and on days 2, 6, 10, 15, and 20 after the start of the injections.

Effect of rhLIF on various organ weights and histological examinations One monkey from the control group and one from the 80 μg/kg/day group were killed on day 15. Various organs including lung, heart, thymus, liver, spleen and kidney were weighed and histological examinations were performed on cut sections stained with hematoxylin and eosin.

RESULTS

Changes in peripheral blood rhLIF increased the blood platelet counts in monkeys at doses higher than 5 μg/kg/day (Fig. 1A). Platelet counts increased by 1.5- to 2-fold on day 15 compared with the control group, and returned to pre-treatment levels on day 30 in the 5 and 20 μg/kg/day groups. In the 80 μg/kg/day group, the platelet counts reached a maximal level on day 20 and exceeded the pre-treatment level even on day 30. No remarkable differences were detected in WBC counts in rhLIF-injected monkeys compared with the control group (Fig. 1B). RBC counts decreased from day 10 in the 80 μg/kg/day group, and returned to the control level immediately after the cessation of rhLIF (Fig. 1C).

Effect of rhLIF on bone marrow findings Relative hyperplasia of myeloid cells was noted in the 80 μg/kg/

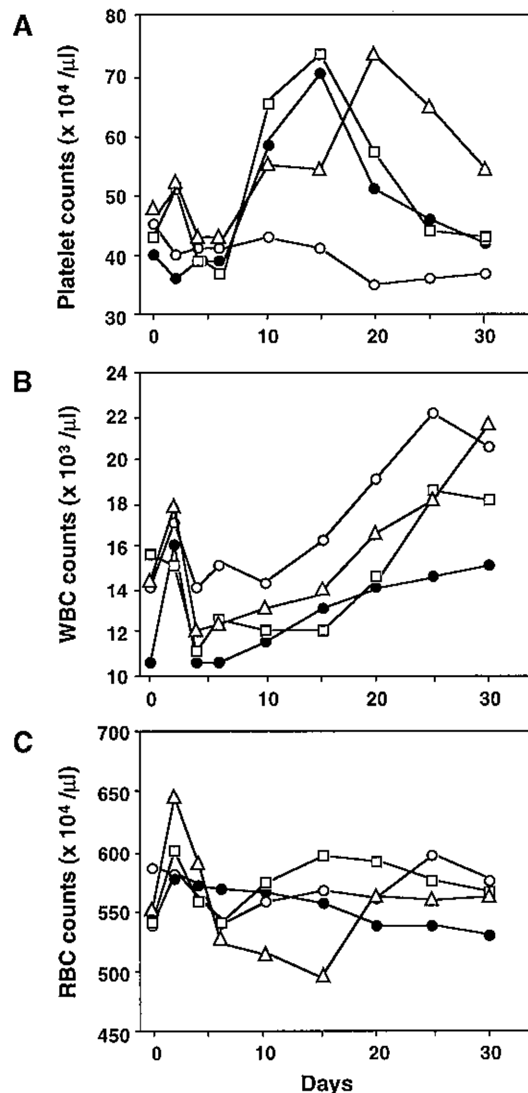

Fig. 1. Effect of rhLIF on platelet, WBC and RBC counts in cynomolgus monkeys. rhLIF (5, 20 or 80 μg/kg/day) was administered to monkeys subcutaneously twice a day for 14 days. PBS (−)+1% heat-inactivated monkey serum was used for the control group. Blood samples were obtained from monkeys on days 0, 2, 4, 6, 10, 15, 20, 25 and 30, and blood cell counts were examined. A, platelet counts; B, WBC counts; C, RBC counts. ○, control; ●, 5 μg/kg/day; □, 20 μg/kg/day; △, 80 μg/kg/day. Each point shows the mean value of two or three monkeys.

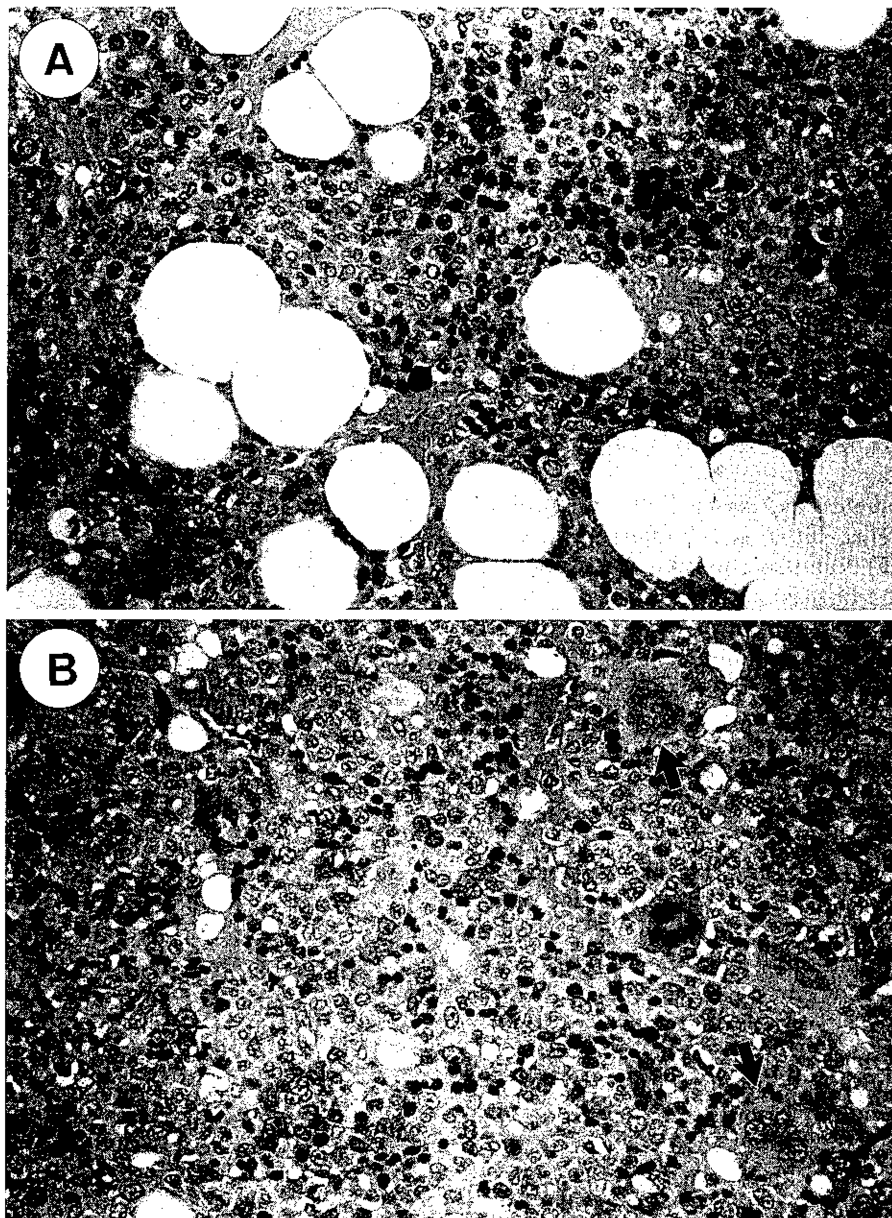

Fig. 2. Histological findings of bone marrow sections from control and rhLIF-injected monkeys killed on day 15. A control monkey (A) and a rhLIF-injected monkey (dose, 80 μg/kg/day) (B). Megakaryocytes indicated by arrows were detected in bone marrow of the rhLIF-injected monkey. Hematoxylin and eosin stain; original magnification, ×100.

day specimen compared with the control group. There was a tendency for the megakaryocyte counts in the 80 μg/kg/day group to increase; however, it was not significant (Fig. 2).

Effect of rhLIF on blood chemical parameters and CRP Serum albumin and T-Cho levels decreased on day 15 in the rhLIF-treated monkeys (Table I). Serum CRP levels increased to maximal values on days 4–6 in a dose-dependent manner in the rhLIF-injected monkeys. The maximal values were retained during the rhLIF-injection period, and then returned to pre-treatment levels immediately after the cessation of rhLIF treatment (Fig. 3).

Weight loss in rhLIF-injected monkeys Body weight decreased by approximately 10% in the monkeys receiving 80 μg/kg/day rhLIF. There was a tendency for body weight to decrease at the doses of 5 and 20 μg/kg/day;

Table I. Effect of rhLIF on Blood Chemical Parameters in Cynomolgus Monkeys

Parameter	Dose of rhLIF (μg/kg/day)			
	0	5	20	80
GOT (IU/liter)	25	21	31	31
GPT (IU/liter)	36	17	22	15
LDH (U/liter)	336	274	305	338
Total protein (g/dl)	8.7	9.0	8.8	7.8
Albumin (g/dl)	4.6	4.3	4.1	3.0
T-Cho (mg/dl)	201	147	ND	100
TG (mg/dl)	84	61	ND	72
BUN (mg/dl)	22	17	12	20
Creatinine (mg/dl)	0.85	0.96	0.84	0.81
Ca (mg/dl)	10.3	10.0	9.6	8.9

Monkeys were injected with rhLIF for 14 days. Blood samples were obtained on day 15 and chemical parameters were measured. Each value represents the mean of two or three monkeys.
ND, not determined.

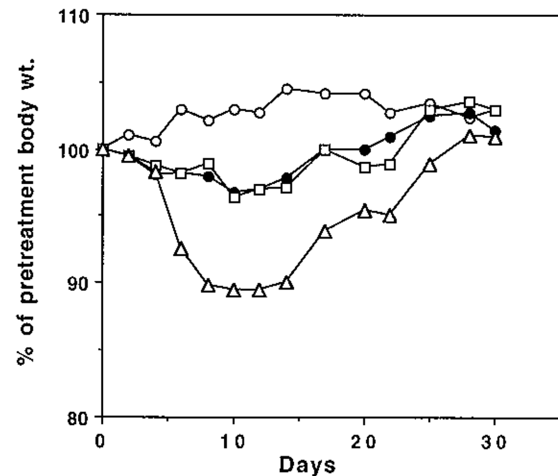

Fig. 4. Effect of rhLIF on body weight in cynomolgus monkeys. Body weight was monitored every day up to day 30 from the start of the injections. ○, control; ●, 5 μg/kg/day; □, 20 μg/kg/day; △, 80 μg/kg/day. Each point represents the mean value of two or three monkeys.

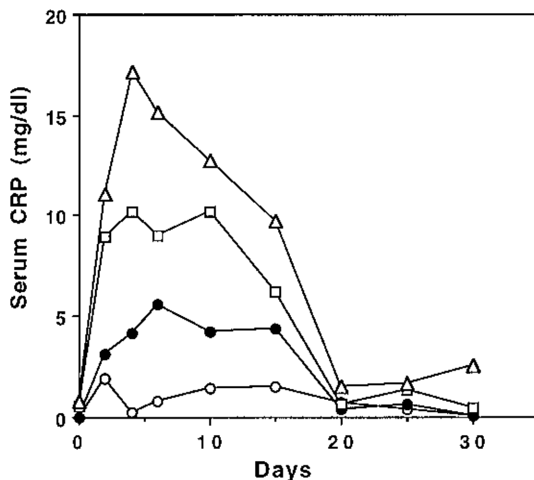

Fig. 3. Serum C-reactive protein (CRP) levels in rhLIF-injected monkeys. Blood samples were drawn from monkeys on days 0, 2, 4, 6, 10, 15, 20, 25 and 30. Serum CRP levels were measured by means of an immunoassay system specific for CRP. ○, control; ●, 5 μg/kg/day; □, 20 μg/kg/day; △, 80 μg/kg/day. Each point shows the mean value of two or three monkeys.

Table II. Effect of rhLIF on Body Weight and Organ Weight in Cynomolgus Monkeys

Organ	Control	LIF: 80 μg/kg
Body weight (kg)	3.13	2.22
Lung (g)	16.27	13.66
Heart (g)	11.08	8.95
Thymus (g)	2.37	0.12
Liver (g)	75.02	72.72
Spleen (g)	2.05	7.71

One monkey from the control group (No. 4) and one from the LIF-injected group (No. 7) were killed on day 15.

however, it was not significant. After the cessation of rhLIF injection, body weight gradually increased, and then recovered to control levels by 30 days from the start of the injections (Fig. 4).

Serum LIF levels in LIF-injected monkeys Serum LIF could not be detected in the control monkeys or the 5 and 20 μg/kg/day rhLIF-injected monkeys. In one monkey receiving 80 μg/kg/day, 50–100 pg/ml of LIF could be detected during the injection (data not shown).

Various organ weights and histological findings in LIF-injected monkeys Macroscopically, a remarkable loss of adipose tissue and prominent splenomegaly were detected in the rhLIF-injected monkey at the dose of 80 μg/kg/day. The spleen weight was 3.5 times heavier than in the control group. A remarkable reduction of organ weight was also detected in the heart and thymus, probably due to loss of adipose tissue (Table II). Histologically, promoted megakaryopoiesis was detected in the spleen; however, no significant changes could be detected in other organs of the rhLIF-injected monkeys.

DISCUSSION

We previously reported that LIF could be a causative factor for cancer cachexia syndrome based on the following observations: (1) LIF possesses potent activity to

inhibit lipoprotein lipase,[11] and (2) three cancer cell lines producing a large amount of LIF induced severe cancer cachexia syndrome in tumor-bearing nude mice.[17, 18] The results of our independent study also suggested that LIF produced by cancer cell lines might play a role in the development of thrombocytosis in mice (manuscript in preparation). In the present study, we attempted to elucidate whether rhLIF can induce cancer cachexia and thrombocytosis in cynomolgus monkeys.

Recombinant hLIF was synthesized by expression of rhLIF (1–180) protein in *E. coli*. The purity of LIF protein was found to be over 95%, and the *N*-terminal amino acid sequence of rhLIF (1–180) was confirmed to be identical to that of authentic LIF. rhLIF (1–180) was revealed to possess appropriate biological activity, when evaluated by colony-forming assay with CMK cells.[19]

Our present study showed that body weight decreased by approximately 10% in monkeys receiving 80 μg/kg rhLIF. There was a slight tendency for decreased body weight in the 5 and 20 μg/kg rhLIF-injected monkeys. Mayer *et al.*[20] also reported that an approximately 10% weight loss was observed in rhesus monkey models injected with 50 μg/kg of rhLIF, although metabolic abnormalities in this experiment were not reported. Farese *et al.*[21] demonstrated the shorter duration of thrombocytopenia in LIF-treated irradiated monkeys, but they did not refer to weight loss. Some researchers have reported abnormalities of lipid metabolism, especially decreased lipogenesis and increased lipolysis, in cancer cachexia models. LIF is well known to inhibit lipoprotein lipase (LPL), which contributes to the synthesis of lipids, as we previously reported.[11] In the present study, we did not examine LPL activity in LIF-treated monkeys, and so could not confirm a direct relationship of LPL-inhibiting activity with weight loss in monkeys. However, we did observe a prominent loss of adipose tissue in a monkey injected with rhLIF. These results suggest that LIF could be a causative factor in increasing lipolysis and developing cachexia syndrome in primates.

With regard to hematopoiesis, platelet counts increased by approximately two-fold at doses over 5 μg/kg rhLIF. No significant effect was detected on WBC or RBC counts. The increase in platelet counts in rhLIF-injected monkeys was considered to be due in part to the increase of megakaryocytes in the bone marrow and particularly in the spleen. The macroscopic findings showed a remarkable splenomegaly, probably due to extramedullary hematopoiesis. These results were similar to those previously reported with interleukin (IL)-6[22] and IL-11.

The c-mpl ligand, or thrombopoietin (Tpo), was recently isolated and shown to have potent thrombopoietic activity in an *in vivo* model. Farese *et al.*[23] reported a 6-fold increase in platelet count in rhesus monkeys injected with 25 μg/kg/day of c-mpl ligand. However, the species of monkey differed from ours.

Recently many studies have been done in the field of cytokine signal transduction systems. Three kinds of thrombopoietic cytokines, IL-6, IL-11 and LIF share gp130 as a common transducer and use some specific JAK (Janus kinase)-STAT (signal transducers and activators of transcription) systems. The c-mpl ligand, Tpo, is reported to use a different hematopoietin receptor from that of cytokines sharing gp130 receptor, but has some common JAK-STAT-mediating signal transduction systems.[24] However, the signal transduction pathway specific for biological activities including thrombocytosis or cachexia syndrome has not yet been established.

Finally, from the viewpoint of the clinical application of LIF, we consider that a suboptimal dose of LIF which can stimulate thrombopoiesis without decreasing body weight merits clinical study.

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