Pharmacological Activity of Chemically Modified Subfragment from Human Serum IgG. XI. Increment of Tumor Necrosis Factor (TNF) Activity in Mouse Sera by Carboxamide-Methylated Light Chain

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Intravenous administration of the carboxamide-methylated light chain (G_1L) from human serum IgG induced tumor necrosis factor (TNF), one of the inflammatory cytokines, in the serum of mice. Human serum IgG, however, showed no induction of TNF in the serum. G_1L showed a potent anti-inflammatory effect in carrageenin-induced paw edema, and the anti-inflammatory effect of G_1L was significantly blocked by the concomitant administration of anti-TNF antiserum, but TNF alone had no anti-inflammatory effect.

Keywords human serum IgG; carboxamide-methylated light chain; anti-inflammatory activity; carrageenin-induced paw edema; L-929 cell cytotoxicity assay; tumor necrosis factor (TNF)

We previously reported that chemically modified subfragments from human serum IgG has anti-inflammatory effects,¹⁾ while intact IgG did not. Among the fragments, the carboxamide-methylated light chain (G_1L , which corresponded to Fr.I-L in our previous paper¹⁾) had the most potent anti-inflammatory activity in several experimental animal models of acute inflammation.

Recently, we found that intravenous administration of G_1L increased the number of granulocytes in blood and the expression of immunoglobulin on the surface of lymphocytes (data not shown). Therefore, we considered that G_1L might activate the immune system leading to systemic inflammation-like symptoms such as ontogenic inflammation.²⁾

Tumor necrosis factor (TNF), a central mediator of acute inflammation, is induced by infections, tissue destruction, tumor growth,³⁾ and lipopolysaccharide (LPS) of some bacteria.⁴⁾ TNF has been known to cause tumor necrosis and inflammation in its physiological roles.⁵⁾ In this paper, we will show that G_1L induces TNF production and discuss the relationship between the TNF induction and the anti-inflammatory effect by G_1L .

MATERIALS AND METHODS

Materials Commercially available human serum IgG (Globulin-Midori) and OK-432 were generously supplied by Green Cross Co., Ltd. (Osaka) and Chugai Pharmaceutical Co. (Tokyo), respectively. Recombinant human tumor necrosis factor- α (h-rTNF- α) was purchased from R & D Systems, Inc. (Minneapolis, MN, U.S.A.), recombinant mouse tumor necrosis factor- α (m-rTNF- α) and polyclonal rabbit anti-mouse TNF- α (for neutralization) from Genzyme Co. (Cambridge, MA, U.S.A.), and actinomycin D from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were the purest grade available.

Cell Line L-929 cells were purchased from Riken Cell Bank (Ibaragi), and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), 1.2 mg/ml sodium bicarbonate,

100 U/ml penicillin G and 100 μ g/ml streptomycin sulfate at 37 °C in 5% CO₂.

Mice Female ICR mice (6-week-old) weighing 25–28 g and male Balb/c mice (6-week-old) weighing 23–25 g were supplied by Japan SLC, Inc. (Hamamatsu). The mice had free access to food and water until the day before the experiments.

Preparation of G_1L G_1L was prepared as described previously.¹⁾ Briefly, human serum IgG was purified by DE-23 (Whatman BioSystems, Ltd., Maidstone, UK) column chromatography to obtain the IgG₁-rich fraction (Fr.I) and the IgG₂-rich fraction. Fr.I was reduced with dithiothreitol and carboxamide-methylated with iodoacetamide, and separated into light (G_1L) and heavy chains by Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography in the presence of sodium dodecyl sulfate (SDS). SDS was removed from each fraction by Dowex 1-X8 (Dow Chemical Company, Michigan, MI, U.S.A.) column chromatography. G_1L was subjected to bioassay after dialysis and lyophilization.

TNF Induction by G_1L *in Vivo* G_1L (0.01, 0.1, 1 mg/kg) was administered into the tail vein of ICR mice. The mice were anesthetized with ether, and whole blood was obtained 1 h after the administration. Sera were prepared by centrifugation at 1500 $\times g$, 4 °C for 15 min after they had been left to clot for 30 min at room temperature, and stored at –80 °C until use.

TNF Induction by G_1L *in Vitro* Splenocytes of Balb/c mice (1×10^6 cells/ml) were cultured with G_1L for 1 or 10 h in RPMI medium supplemented with 10% FBS, 1.2 mg/ml sodium bicarbonate, and antibiotics at 37 °C in 5% CO₂. TNF activity of *in vitro* samples were determined by L-929 cell cytotoxic assay as described.⁶⁾

Determination of TNF Activity of *in Vivo* Samples TNF activity of *in vivo* samples was determined by L-929 cell cytotoxic assay with slight modification. Briefly, L-929 cells (6×10^4 cells/100 μ l of the medium) were cultured with diluted serum samples or h-rTNF- α as a standard in a flat-bottomed 96-well multiplate for 18 h in the presence of actinomycin D. In the assay system, for the standard using h-rTNF, normal mouse serum at a final concentra-

tion of 6.2% treated with anti-mouse TNF- α antibody was added to the wells to eliminate the activity of the growth factors in mouse serum samples. Killing of L-929 cells was assessed by uptake of crystal violet dye. TNF activity (U/ml) was calculated from the standard curve.

Carrageenin-Induced Paw Edema in Mice G₁L (1 mg/kg), anti-mouse TNF- α (2 μ l/mouse, this amount can neutralize 2×10^3 U of mouse TNF), OK-432 (3 KE/mouse), and m-rTNF- α (1×10^3 U/mouse) were intravenously administered into ICR mice. Thirty minutes later, carrageenin (1.5%, 20 μ l) and saline were subcutaneously injected into right and left hind paws of mice, respectively. Three hours after the carrageenin injection, the difference in thickness between the paws was measured by a dial thickness gauge (Mitutoyo Co., Ltd., Tokyo) and the intensity of the edema was expressed as swelling percentage.

Statistics Student's *t*-test was employed to assess the statistically significant difference between the control and sample-administered groups.

RESULTS

TNF Induction in Mouse Sera after Intravenous Administration of G₁L L-929 cell cytotoxic activity in mouse sera was the highest 1 h after intravenous administration of G₁L (1 mg/kg), and thereafter the activity decreased rapidly (Fig. 1). L-929 cells are known to be injured by IL-1 and TNF. The L-929 cell cytotoxic activity in the sera of G₁L-administered mice was neutralized by anti-mouse TNF- α antiserum to less than

Fig. 1. L-929 Cell Cytotoxic Activity in Mouse Sera after Intravenous Administration of G₁L

G₁L was administered at a dose of 1 mg/kg. Each column represents mean $\pm$ S.E. (N=6). Significant difference from the control group: a) $p < 0.05$.

TABLE I. Neutralization of L-929 Cell Cytotoxic Activity in Sera of G₁L-Administered Mice by Anti-TNF- α Antiserum

Treatment	Dose (mg/kg)	TNF activity (U/ml)	
		No addition	Anti-TNF- α addition
Control	—	1.80 $\pm$ 0.39	N.D.
G ₁ L	1	6.92 $\pm$ 1.41 ^{a)}	N.D.

Each value represents mean $\pm$ S.E. (N=6). N.D.: not detectable. Significant difference from the control group: a) $p < 0.05$.

0.39 U/ml, the detectable limit of this assay system for *in vivo* samples (Table I). This result indicated that the L-929 cell cytotoxic substance in the sera of G₁L-administered mice was TNF.

Activity of G₁L and Fr.I to Induce TNF TNF activity in mouse sera was evaluated 1 h after intravenous administrations of G₁L (1 mg/kg) and Fr.I (1 mg/kg) (Fig. 2). The TNF activity was significantly increased by the administration of G₁L (1 mg/kg), but not by Fr.I

Fig. 2. Effects of G₁L and Fr. I on TNF Induction in Mouse Sera

Each column represents mean $\pm$ S.E. (N=6). Significant difference from the control group: a) $p < 0.001$.

Fig. 3. Dose-Dependent Effect of G₁L on TNF Induction in Mouse Sera

Each column represents mean $\pm$ S.E. (N=5—6). Significant difference from the control group: a) $p < 0.01$, b) $p < 0.001$.

Fig. 4. Effects of G₁L and/or Anti-TNF- α Antiserum on Carrageenin-Induced Paw Edema in Mice

G₁L was administered intravenously at a dose of 1 mg/kg. Ab: anti-mouse TNF- α antiserum. Each column represents mean $\pm$ S.E. (N=4—5). Significant difference from the control group: a) $p < 0.001$. Significant difference from the G₁L-treated group: b) $p < 0.05$.

Fig. 5. Effects of G₁L, OK-432 and TNF-α on Carrageenin-Induced Paw Edema in Mice

G₁L (1 mg/kg), OK-432 (3 KE/mouse) and TNF-α (1 × 10³ U/mouse) were administered intravenously. Each column represents mean ± S.E. (N=4–6). Significant difference from the control group: a) *p* < 0.05.

TABLE II. Production of TNF by Splenocytes Cultured with G₁L, OK-432 and LPS

Treatment	Concentration	TNF activity (U/ml)	
		1 h	10 h
Control	—	N.D.	N.D.
G ₁ L	100 μg/ml	N.D.	2.98
OK-432	0.1 KE/ml	N.D.	13.55

Cells (1 × 10⁶ cells/ml) were cultured for 1 or 10 h with each sample. N.D.: not detectable.

(1 mg/kg).

Dose-Dependency for G₁L to Induce TNF Induction of TNF in mouse sera was evaluated 1 h after intravenous administration of G₁L at various doses of 0.01, 0.1, 1 mg/kg. As shown in Fig. 3, G₁L increased serum TNF in a dose-dependent manner.

Contribution of TNF to Anti-inflammatory Activity of G₁L We evaluated whether TNF induced in the sera of G₁L-administered mice was important in the anti-inflammatory effect of G₁L (Fig. 4). G₁L significantly inhibited carrageenin-induced paw edema in mice, a model of acute inflammation. The concomitant administration of anti-mouse TNF-α antiserum, in an amount adequate to neutralize 2 × 10³ U of TNF, significantly blocked the anti-inflammatory effect of G₁L. However, the antiserum alone had no effect on carrageenin-induced paw edema.

We evaluated to effects of m-rTNF-α and OK-432, known as a TNF inducer, on carrageenin-induced paw edema in mice (Fig. 5). Neither TNF-α nor OK-432 inhibited the inflammation.

TNF Production by Mouse Splenocytes Cultured with G₁L We assayed TNF production by mouse splenocytes stimulated with G₁L *in vitro*. Control indicated spontaneous TNF secretion from the splenocytes. Both G₁L and OK-432 used as a positive control increased the TNF level in the culture supernatant at 10 h after the addition.

DISCUSSION

In this study, we focused on the activity of G₁L to induce TNF, a central mediator of inflammation, because G₁L had shown potential to stimulate the immune system (data not shown). TNF was rapidly induced by G₁L *in vivo* and *in vitro*. We previously showed the potent anti-inflammatory effects of G₁L.¹⁾ Interestingly, anti-mouse TNF antiserum significantly suppressed the anti-inflammatory effect of G₁L in carrageenin-induced paw edema, suggesting that one of the anti-inflammatory mechanisms of G₁L might be the TNF induction, although this is contrary to the general agreement of TNF as an inflammatory mediator.⁵⁾ It is possible that TNF plays a key role not only in the initiation of inflammation but also in the recovery of the tissue from the inflammation. For instance, TNF induces anti-inflammatory substances, glucocorticoids and acute phase reactants such as C-reactive protein 12–24 h after the stimulation.³⁾ However, it is difficult to postulate that the anti-inflammatory substances induced by TNF were the mechanism for the anti-inflammatory activity of G₁L, because of the time course of G₁L anti-inflammatory action. G₁L suppressed the acute phase inflammation. TNF might down-regulate some adhesion molecules on neutrophils, thus showing the anti-inflammatory effect. Neutrophils expressing LECAM-1 (leukocyte endothelial cell adhesion molecule-1) initiate rolling on endothelial cells in the first stage of acute inflammation. The neutrophils then adhere to the endothelial cells with high avidity integrins, and finally migrate into the inflammatory tissue. Recently, Miguel *et al.* reported that TNF down-regulates the expression of some adhesion molecules such as CD43, CD44 and LECAM-1 on leukocytes along with up-regulation of the integrin expression.⁷⁾ Moreover, Watson *et al.* found that the ligand of LECAM-1 receptor-chimera molecule blocks the infiltration of neutrophils in acute inflammation.⁸⁾ From this evidence, we considered that when LECAM-1 on neutrophils was rapidly shed by the stimulation of TNF, the neutrophils were unable to begin rolling during the first step of inflammation. Indeed, LECAM-1 was slightly down-regulated after intravenous administrations of both G₁L and TNF (data not shown). However, we concluded that the down-regulation of LECAM-1 had little contribution to the anti-inflammatory effect of G₁L, because TNF, which is a down-regulator, had no effect on the inflammation (Fig. 5). More research is required to clarify the correlation between the TNF induction and the anti-inflammatory effect of G₁L.

The anti-inflammatory effect of G₁L was significantly blocked by concomitant administration of anti-mouse TNF antiserum (Fig. 4). Our results (Table II) suggest that both G₁L and OK-432 are TNF inducers for macrophages. OK-432 was a much stronger inducer of TNF than G₁L, though the former showed less activity in its anti-inflammatory effect (Fig. 5). Therefore, it is not likely that G₁L shows the anti-inflammatory effect through TNF which it induces. We are very interested, however, that TNF was induced by G₁L but not by IgG. The precise mechanism of TNF induction by G₁L remains unknown.

TNF is known to be produced by various kinds of cells, mainly by macrophages.⁹⁾ G₁L also induced TNF *in vitro* (Table II); therefore, TNF induction in the sera might be derived from macrophages directly activated by G₁L. TNF was produced by splenocytes cultured for 10 h *in vitro* (Table II). The reason TNF was not induced for 1 h but 10 h *in vitro* would be that a small number of cells was not enough to produce much detectable TNF. Moreover, the reason TNF induced *in vivo* was rapidly reduced would be that many receptors on cells, binding proteins, and inhibitors were involved.

In conclusion, G₁L specifically and rapidly induced TNF *in vivo* and *in vitro*, though the mechanism of this induction remains unknown. We plan to study the interaction between G₁L and macrophages to elucidate the mechanism by which TNF is induced.

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