INVOLVEMENT OF 26-kDa MEMBRANE-BOUND TUMOUR NECROSIS FACTOR PRECURSOR IN BIDIRECTIONAL FEEDBACK REGULATION ON 17-kDa TUMOUR NECROSIS FACTOR PRODUCTION AFTER STIMULATION BY LIPOPOLYSACCHARIDE

Yoshiyuki Tanabe,[1] Chie Kohchi,[2] Namiko Kitahara-Tanabe,[3] Den-Ichi Mizuno,[4] Gen-Ichiro Soma[5,6]

The authors have previously shown that 26-kDa membrane-bound tumour necrosis factor precursor (proTNF) on the cell-surface of primed human monocytic cell line THP-1 is involved in positive feedback regulation of lipopolysaccharide (LPS)-dependent TNF-production. Here, we provide direct evidence for modulation of responsiveness of the THP-1 cells against LPS by membrane-bound proTNF. When THP-1 cells were cocultivated with a heterogeneous cell line (proTNF/3T3 cells) which constitutively expressed membrane-bound proTNF, LPS-dependent TNF-production by THP-1 cells was significantly suppressed and the normal level was restored by the presence of anti-TNF antibody during cocultivation. The proTNF-3T3-induced decline of TNF-production of THP-1 was observed primarily at the mRNA level, although no difference was observed in the mRNA level of interleukin 1β, another LPS-inducible cytokine. These results suggest that proTNF could also be involved in the negative feedback regulation of LPS-dependent TNF-production through cell-to-cell contact. The augmentation of LPS-dependent TNF-production accompanied by the production of endogenous proTNF induced by exogenous agent was inhibited by protein kinase C inhibitor, whereas proTNF/3T3-induced suppression of TNF-production could not be restored to the normal level. It thus seems possible that proTNF might act on macrophages as a bidirectional regulator of its production by THP-1 cells depending on co-induced signals.

Tumour necrosis factor (TNF) is a member of the inflammatory cytokine network which is believed to regulate various inflammatory states in the body. Using recombinant material, it has been well elucidated that mature TNF with a molecular size of 17 kDa shows pleiotropic activities related to a variety of inflammatory processes seen in adult (for review, see Ref. 1).

From the [1]Department of Pharmacology, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan; [2]Radioisotope Center, Hiroshima University, Higashi-Hiroshima, Japan; [3]2nd Department of Surgery, St Marianna University, School of Medicine, Kawasaki, Japan; [4]Institute of Microbial Chemistry, Tokyo, Japan; [5]Institute of Medical Science, St Marianna University, Kawasaki, Japan; [6]Department of Molecular Medicine, Takano Hospital, Kumamoto, Japan

Correspondence to: Gen-Ichiro Soma, Department of Molecular Medicine Takano Hospital 4-2-88 Obiyama, Kumamoto 862, Japan

Received 5 March 1997; accepted for publication 27 June 1997

1043–4666/98/020082 + 11 $25.00/0/ck970266

KEY WORDS: lipopolysaccharide/membrane bound TNF precursor/THP-1 cells/tumour necrosis factor

Significance of an inflammation-like state which we consider to be a prototype of inflammation is related to TNF-production, either by a regulatory mechanism of embryogenesis or a restorative mechanism of physiologically distorted states of adult and has been documented.[2] In the prototype of inflammation, the 26-kDa membrane-bound TNF precursor (proTNF) may be the main molecule exerting the biological function to maintain the general homeostasis, because in adult free 17-kDa TNF is barely detectable at the normal[3,4] or primed stage for TNF-production,[5–7] although a significant amount of TNF mRNA is detected in these stages.[3,4,7] Moreover, not 17-kDa TNF but 26-kDa proTNF is predominantly expressed during normal embryogenesis of mice.[8] In these cases, proTNF acts only on neighbouring cells through cell-to-cell contact and its direct effect is localized. At the later stage, its secondary effect pervades the whole body and, with free TNF, it orchestrates an inflammatory cytokine network.[9]

As an experimental system to investigate the biological significance of proTNF at the primed stage, we previously reported[10] that interferon (IFN)-γ-

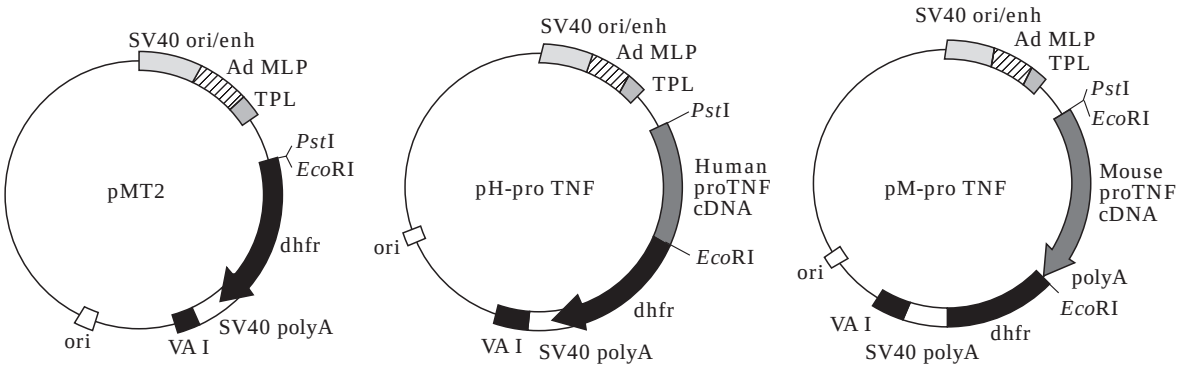

Figure 1. Schematic representation of the 26-kDa proTNF expression construct.

cDNA fragments encoding either human or mouse proTNFs were ligated with mammalian expression vector pMT-2 having a modified adenovirus major late promoter.[13] AU-rich motif in 3′-UTRs of both human and mouse TNF genes involved in the stability of their mRNA[14] have been removed from constructs.

primed THP-1 cells expressing membrane-bound proTNF can produce a larger amount of TNF than is produced by normal THP-1 in response to the addition of lipopolysaccharide (LPS). When IFN-γ and anti-TNF monoclonal antibody (mAb) were applied simultaneously to THP-1, the priming effect of IFN-γ was specifically abolished. Therefore, it can be postulated that TNF precursor expressed at the primed stage may stimulate THP-1 to produce a larger amount of mature TNF in response to the addition of LPS. This function seems to be a positive feedback mechanism of TNF-production by its own membrane-bound precursor, probably through cell-to-cell contact. However, there has been no direct evidence that proTNF is the sole molecule on the cell surface to have such an autoregulatory effect on its own production. We therefore sought to show a direct effect of proTNF on TNF-producibility of THP-1 cells in this study.

The effect of proTNF expressed on the surface of a heterogenous fibroblast cell line, NIH3T3, on LPS-dependent mature TNF-production by THP-1 cells will be reported here. LPS-dependent TNF-production by THP-1 cells was significantly diminished after coculture with the fibroblast cell line expressing proTNF. ProTNF on the cell-surface is thus likely to be involved in regulating either positive or negative feedback of its own production, depending upon the co-induced signal transduction pathway in the THP-1 cells.

RESULTS

LPS-inducible TNF-production by THP-1 cells was decreased after contact with a heterogenous cell line expressing proTNF

ProTNF is a membrane integrated protein that exposes its functional domain corresponding to the mature TNF outside of the cell.[11] It is therefore believed that proTNF exhibits its biological activities to neighbouring cells through cell-to-cell contact.[11,12] As an experimental system to simulate a mode of action of proTNF in a homogenous culture of primed THP-1 cells,[10] we cocultivated heterogenous cells expressing proTNF and THP-1 cells.

To establish the cell line expressing proTNF permanently, the plasmid DNAs for the expression of either human (h) or mouse (m) proTNF (Fig. 1) were cotransfected with pMC1neo-polyA into a mouse embryonic fibroblastic cell line NIH3T3. The stably transfected cell lines were established and referred to as either hproTNF/3T3 or mproTNF/3T3 cells. Preferential expression of 26-kDa proTNF was detected in an extract from either h or mproTNF/3T3 cells by Western blot analysis (Fig. 2A). Surface expression of 26-kDa proTNF on the h and mproTNF/3T3 cells was also confirmed by a combined procedure of surface biotinylation and immunoprecipitation described by Meier *et al*.[16] The 26-kDa band corresponding to biotinylated proTNF was specifically immunoprecipitated in both cases of h and mproTNF/3T3 cells (Fig. 2B), indicating that proTNFs were actually expressed on the surface of these transfectants. Spontaneous release of mature TNF in the culture supernatant of hproTNF/3T3 cell line was observed (about 50 to 100 units/ml by L929 cytotoxic assay). However, TNF activity in the supernatant of the m-proTNF/3T3 cell line was below the detectable level (less than 0.3 units/ml).

THP-1 cells were seeded onto either h or mproTNF/3T3 monolayers and co-incubated for 24 h. Microscopic examination showed no apparent sign of damage of the monolayer of NIH3T3 transfectant after the start of cocultivation. Suspended cells, mostly THP-1 cells, were then harvested, washed and challenged with LPS. Five hours after this challenge, the amount of TNF in the culture supernatant was measured by RIA.

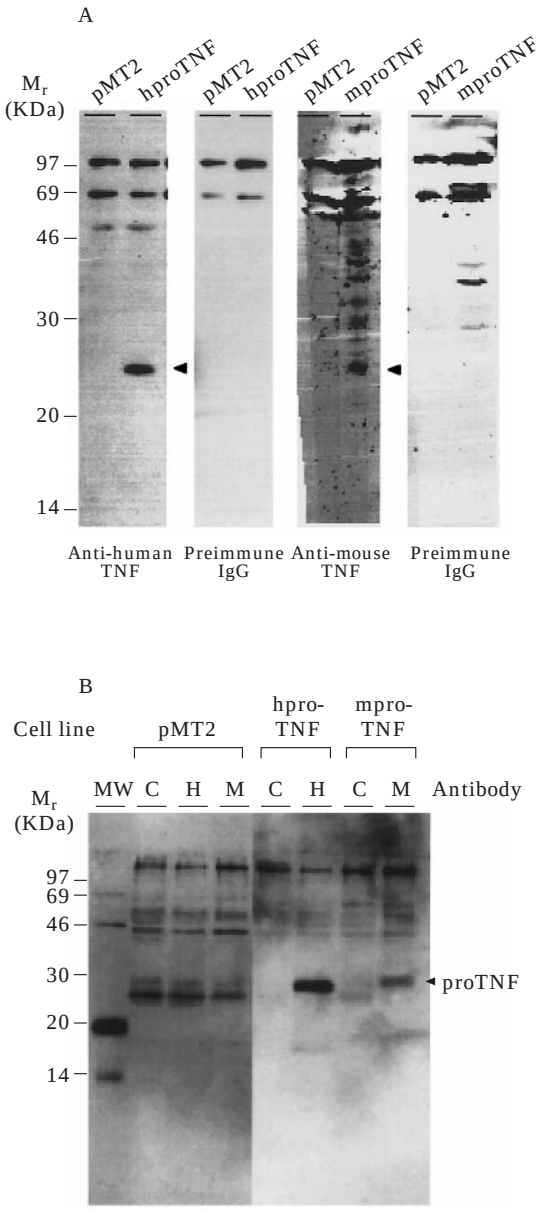

Figure 2. Establishment of the feeder cell lines expressing proTNF.

A: Either control plasmid (pMT-2), human proTNF construct (hproTNF), or mouse proTNF construct (mproTNF) was introduced to NIH3T3 mouse fibroblast cells with pMC1neo-polyA.[15] Permanently transfected cell lines were established by Geneticin (G-418) selection. Cell extract equivalent to 50 μg of proteins was subjected to ECL-Western immunostaining analysis probed with either anti-human or anti-mouse TNF pAbs. B: Either pMT-2/3T3, hproTNF/3T3, or mproTNF/3T3 cells was surface biotinylated using biotin–CNHS-ester according to a previously described method.[16] Cell extracts containing equivalent amounts of protein were separately immunoprecipitated using control rabbit IgG (lane C), anti-human TNF pAb (lane H), and anti-mouse TNF pAb (lane M). The immunoprecipitated materials were then size fractionated, transferred to nitrocellulose filter, then probed with streptavidin–horseradish peroxidase and ECL-Western detection system. MW: biotinylated molecular weight standard.

Unexpectedly, LPS-inducible TNF-secretion was significantly lower than the unprimed THP-1 cells when the cells were pre-exposed to either h or mproTNF/3T3 feeder monolayer (Fig. 3, $P < 0.01$). No decrement in viability of THP-1 cells was observed after cocultivation with either group of proTNF/3T3 cells as judged by trypan blue dye exclusion. In a separate experiment, a monolayer of NIH3T3 transfectant was treated with 1% paraformaldehyde in PBS(−) for 30 min at room temperature to fix the membrane protein.[17] This fixed cell was washed five times with PBS(−), incubated in the medium for 24 h, then washed again five times with PBS(−) prior to cocultivation with THP-1 cells. A similar result was observed when proTNF/3T3 feeder cells were fixed with paraformaldehyde to avoid the spontaneous secretion of mature TNF from them (data not shown).

The suppressive effect of proTNF/3T3 cells on LPS-dependent TNF-production by THP-1 cells completely disappeared when anti-TNF antibodies were added during the cocultivation period (Fig. 4). These results suggest that proTNF on heterogenous cells must play a role in the negative regulation of LPS-dependent TNF-producibility of THP-1 cells through cell-to-cell contact.

Suppression of LPS-dependent TNF-production after contact with proTNF/3T3 cells at the mRNA level

To obtain further insight into the mechanism of suppressive effect of proTNF/3T3 cells on LPS-dependent TNF-production by THP-1 cells, we examined the LPS-dependent expression of mRNA for TNF and IL-1β after cocultivation.

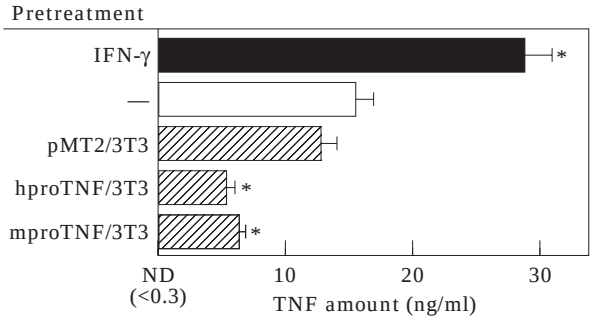

Figure 3. Suppression of LPS-dependent TNF production by THP-1 cells cocultivated with proTNF/3T3 cells.

pMT2/3T3 cells, human proTNF/3T3 (h-proTNF/3T3) cells, and mouse proTNF/3T3 (m-proTNF/3T3) cells (1.5×10^6) were seeded onto gelatin-coated plastic dishes (ϕ60 mm) and were incubated to allow formation of a confluent monolayer. THP-1 cells (5×10^6) were seeded at the density of 1×10^6 cells/ml onto monolayers of various 3T3 transfectants (▨). IFN-γ-primed (■) or unprimed (□) THP-1 was prepared simultaneously as controls. After 24 h, THP-1 cells were recovered, washed, and challenged with LPS (0.1 μg/ml). Five hours after the addition of LPS, culture supernatants were subjected to RIA for mature TNF.[10] Each value with a horizontal bar represents mean ± SE of 15 experiments. Levels of significance were determined by Tukey's multiple comparison test. *$P < 0.01$ vs control (□) value.

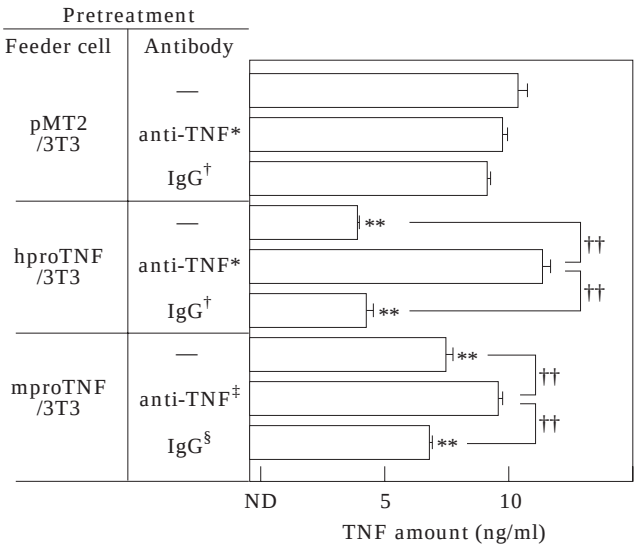

Figure 4. Disappearance of the proTNF/3T3-induced suppression of LPS-dependent TNF-production by anti-TNF Abs.

Anti-TNF Abs were added to the THP-1 versus proTNF/3T3 cocultivation system. After 24 h, cells were washed and challenged with LPS (0.1 μg/ml) for 5 h. The amount of TNF in the culture supernatant was measured by RIA. *anti-human rTNF-α mAb, †nonspecific mouse IgG, ‡anti-mouse rTNF-α pAb, §normal rabbit IgG. Each value with a horizontal bar represents mean ± SE of three experiments. Levels of significance were determined by Tukey's multiple comparison test. $^{**}P < 0.01$ vs pMT2/3T3 without antibody. $\dagger\dagger P < 0.01$ vs either h or mproTNF with anti-TNF Abs.

A strong suppressive effect of hproTNF/3T3 cells on LPS-dependent expression of TNF mRNA by THP-1 was observed, while only marginal suppression was shown by mproTNF/3T3 cells (Fig. 5). However, this is probably significant because pretreatment of THP-1 cells with LPS, which induces a refractory state of macrophages against a secondary challenge of LPS,[18–21] showed a similar degree of suppressive effect of LPS-dependent expression of TNF mRNA (Fig. 5).

In contrast to TNF, no difference was observed for the level of transcription of IL-1β mRNA which is also induced by LPS (Fig. 5), suggesting that proTNF-induced hyporesponsiveness against LPS is specific for TNF gene expression.

Differences in the involvement of protein kinase C in the positive and negative regulation of TNF-production mediated by proTNF

As described,[10] the expression of proTNF during the primed stage of THP-1 is involved in the sensitization against LPS for TNF-production. However, we have also shown in this study that proTNF expressed by the heterogenous cells desensitizes THP-1 against LPS through cell-to-cell contact. Therefore, we assume that these opposite effects of proTNF on LPS-inducible TNF-production might be ascribable to the difference of co-induced signals in the THP-1 cells.

It has been reported that IFN-γ, TNF, and phorbol myristate acetate (PMA) exhibit their biological activities through activation of protein kinase C.[22–24] To discriminate the difference of mechanisms in primed THP-1 itself from that in normal THP-1 cells in contact with proTNF/3T3 cells, cells of the heterogenous cell line, THP-1 were treated with either IFN-γ, TNF, or proTNF/3T3 cells in the presence or absence of protein kinase inhibitor, H7, which has a relatively broader spectrum against serine-threonine kinase including protein kinase C as well as cyclic nucleotide-dependent protein kinases.[25] After extensive washing to remove both the priming agent and H7, THP-1 cells were challenged with LPS and the amount of TNF in the culture supernatant was then measured.

Augmentation of mature TNF secretion by either IFN-γ-primed or TNF-primed THP-1 did not occur in the presence of H7 (Fig. 6A, B), suggesting that activation of the H7-sensitive protein kinase pathways as well as expression of endogenous membrane-bound proTNF[10] is involved in and necessary for the enhanced TNF-production by primed THP-1 cells in response to LPS. H7 did not, however, interact with the suppression of mature TNF secretion by THP-1 cells when in contact with heterogenous proTNF/3T3 cells (Fig. 7), suggesting that H7-sensitive protein kinases are not directly involved in the suppressive effect of proTNF/3T3 cells on LPS-dependent TNF production by THP-1 cells.

We further characterized the H7-sensitive protein kinase activated in IFN-γ-primed THP-1 cells using GF109203X, a highly selective inhibitor of protein kinase C.[26] When GF109203X was added at the beginning of IFN-γ pretreatment, subsequent TNF-production depending on LPS-challenge was significantly diminished, whereas this did not occur with pretreatment with GF109203X alone (Fig. 8). This suggests that H7-sensitive protein kinase involved in the membrane-bound proTNF-mediated augmentation of TNF-production by IFN-γ-primed THP-1 cells may be protein kinase C.

DISCUSSION

We previously proposed that the teleological meaning of inflammation is not simply as a defence mechanism against infection but as a regulatory system of homeostasis of animals in general.[2] We hypothesized that the inflammation-like state characterized by expression of membrane-bound 26-kDa TNF on the surface of somatic cells could be involved in the regulatory mechanism of homeostasis.

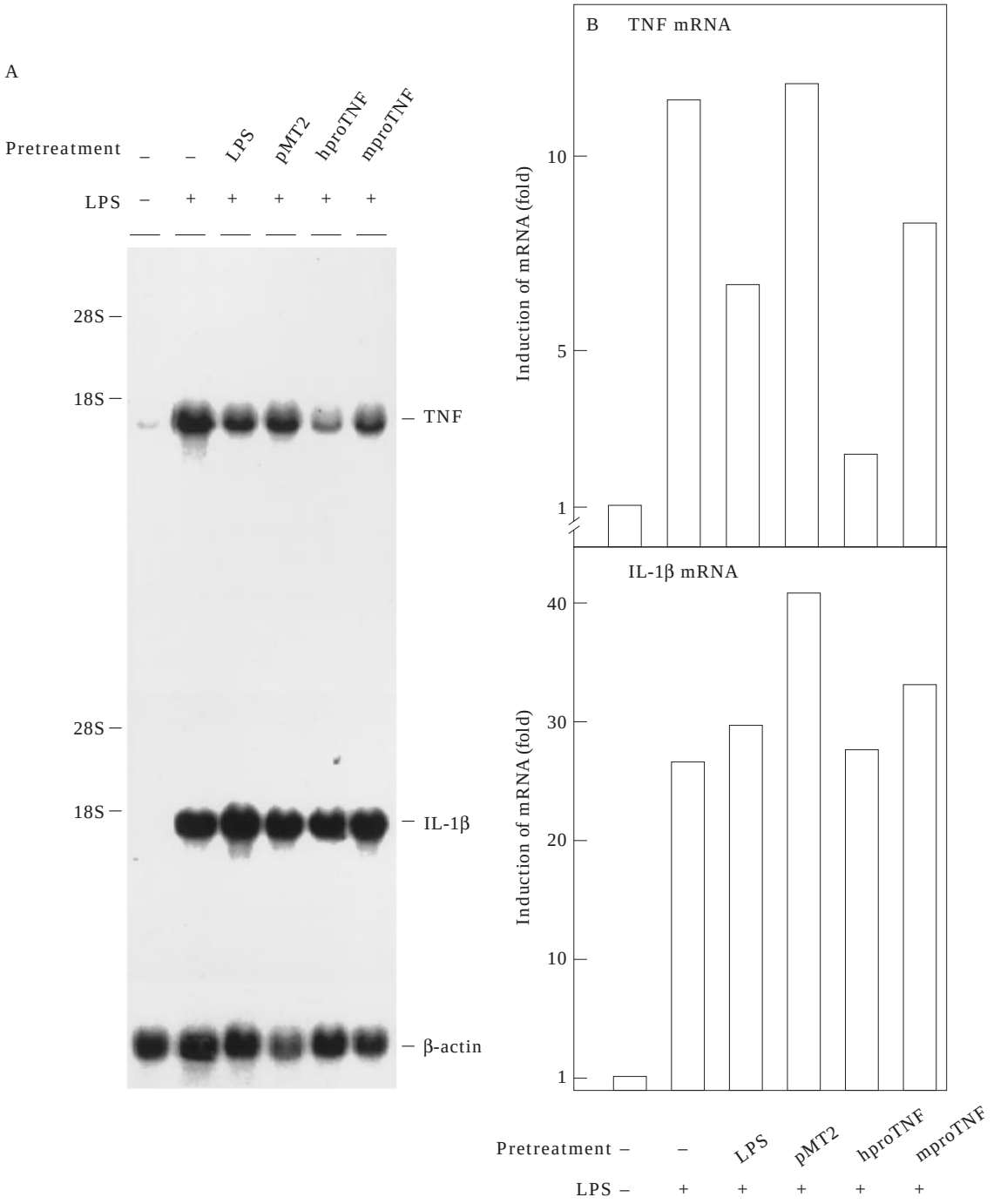

Figure 5. Northern blot analysis of LPS-dependent expression of endogenous TNF and interleukin 1β genes in THP-1 cells after cocultivation with proTNF/3T3 cells.

THP-1 cells were cocultivated with or without various 3T3 transfectants for 24 h as described in Figure 3. LPS-pretreated (0.1 μg/ml) cells were also prepared in parallel as a control experiment inducing the hyporesponsive state of THP-1 cells against secondary LPS challenge. After 24 h, THP-1 cells were recovered, washed, and challenged with LPS (0.1 μg/ml). Total cellular RNA was extracted from these cells and size fractionated by agarose gel electrophoresis. RNA was then blotted onto membrane and hybridized with either ^{32}P-labelled human TNF, IL-1β, or β-actin probes, respectively. A shows autoradiograms of the Northern hybridization experiment and B shows semiquantitative indication of induction of TNF and IL-1β mRNAs in THP-1 cells. Values shown have been normalized with the level of β-actin mRNA in each preparation. Results of a representative experiment are given.

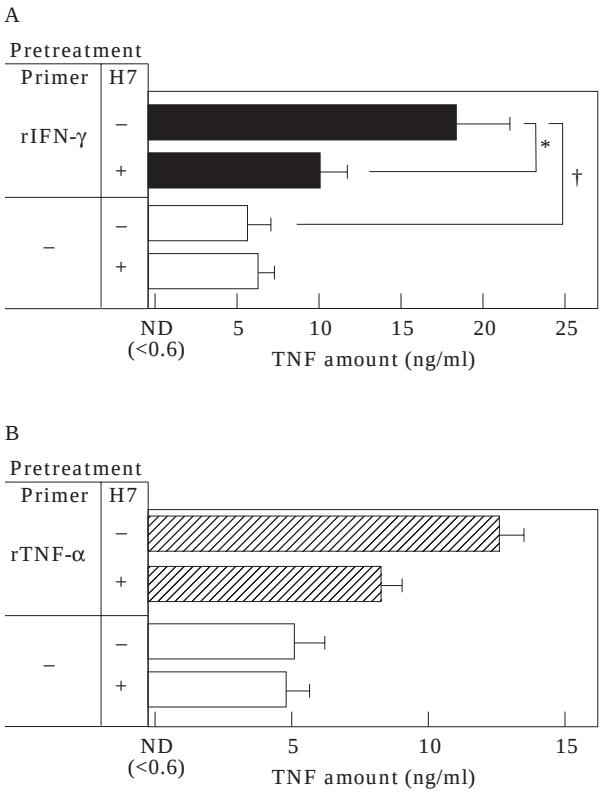

Figure 6. Involvement of H7-sensitive protein kinase in the exogenous cytokine-induced hyper-responsive state of THP-1 cells against LPS.

A: effect of protein kinase inhibitor (H7) on the priming activity of rIFN-γ. THP-1 cells were treated with rIFN-γ (10^3 U/ml, ■) for 24 h in the presence or absence of 50 μM H7. LPS treatment was as described in Figure 3. TNF amount in the culture supernatant was measured by RIA. Each value with a horizontal bar represents mean ± SE of four experiments. Levels of significance were determined by Student's *t*-test. $*P < 0.05$, $\dagger P < 0.01$. B: effect of H7 on the priming activity of rTNF-α. THP-1 cells were treated with rTNF-α (10^3 U/ml, ▨) for 24 h in the presence or absence of 50 μM H7. LPS treatment was as described in Figure 3. TNF amount in the culture supernatant was measured by RIA. Results (mean values ± SD of triplicate measurements) of a representative experiment are given. (□), control.

The pleiotropic function of mature TNF was verified, although we think that proTNF may be a more important form than mature TNF, because only a small amount of the latter was detected in a limited region of embryo,[27] whereas expression of TNF mRNA was observed in various embryonic tissues.[28] Our idea was also strongly supported by the earlier finding of Osawa and Natori[8] in which the actual form of TNF during embryogenesis was the precursor of 26 kDa.

Kriegler and coworkers reported that proTNF exhibits cytotoxic activity against certain transformed cell lines in vitro,[11,12] showing that its activity is similar to mature TNF. Cytotoxic activity of proTNF has been thought to be mediated by the same receptor as that of mature TNF via cell-to-cell contact.[11,12] However, it should be confirmed whether or not proTNF really acts as a regulatory molecule. We have reported that TNF shows a priming activity for its production in vivo by a positive feedback system.[29] Thus, the role of proTNF in the self-regulation of its own producibility is a good model for its regulatory role in the cytokine network.

As a first step, we reported that expression of proTNF is a common event during the priming stage of THP-1 cells regardless of the type of priming agents examined (rIFN-γ, rTNF-α, or PMA).[10] We also reported that proTNF possibly augments the self-reproducibility through an intercellular positive feedback mechanism.[10] However, because the induction of endogenous proTNF was achieved by the addition of an exogenous reagent, various kinds of coinduced signal(s) might also be involved. Furthermore, discrimination between effector and target was ambiguous in the homogenous culture of THP-1 cells. To examine the single-handed role of proTNF on the self-reproducibility in these cells, we produced heterogenous NIH3T3-derived cell lines which express proTNF cDNA constitutively as a defined source of proTNF (h or mproTNF/3T3 cells) (Fig. 2A, B).

Cocultivation with proTNF/3T3 cells did not augment but significantly diminished the

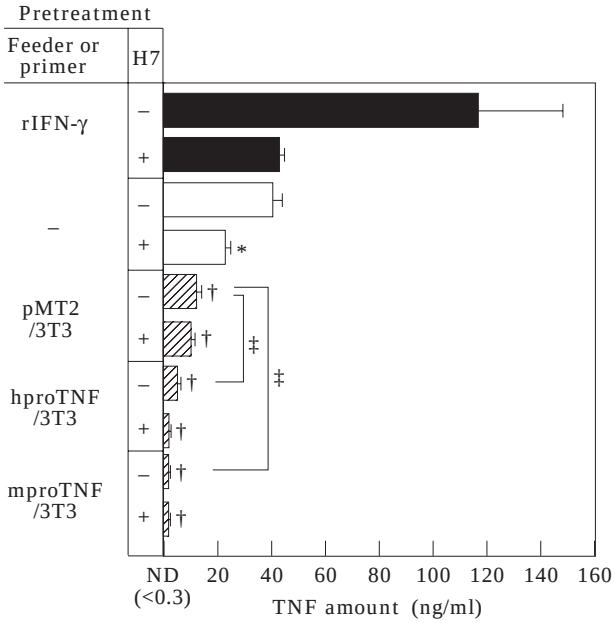

Figure 7. Effect of H7 on the suppression of LPS-dependent TNF-production of THP-1 cells induced by proTNF/3T3 cells.

THP-1 cells were cocultivated with proTNF/3T3 cells (netted bars) in the presence or absence of 50 μM H7. IFN-γ-primed (■) and unprimed (□) THP-1 were prepared simultaneously as controls. LPS treatment was as described in Figure 3. TNF amount in the culture supernatant was measured by RIA. Each value with a horizontal bar represents mean ± SE of four experiments. Levels of significance were determined by Student's *t* test. $*P < 0.05$, $\dagger P < 0.01$ vs control (−, without H7) value. $\ddagger P < 0.01$ vs pMT2/3T3 (without H7) value, respectively.

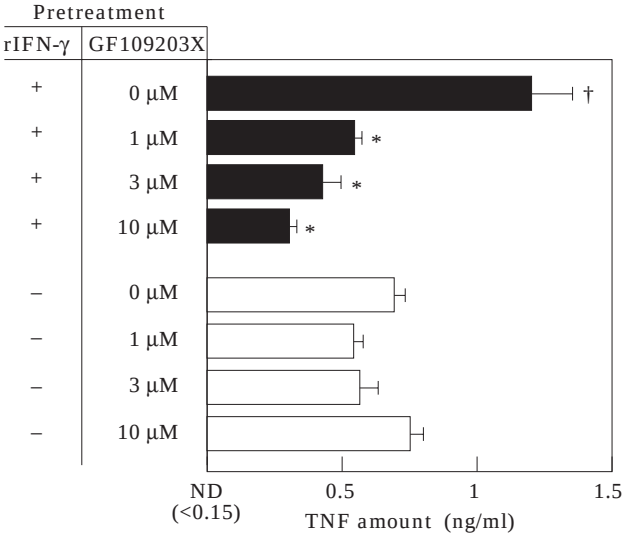

Figure 8. Effect of GF109203X, a selective inhibitor of protein kinase C, on the priming activity of IFN-γ.

THP-1 cells were treated with rIFN-γ (10^3 U/ml, ■) for 24 h in the presence or absence of GF109203X at indicated concentrations. After 24 h, they were recovered, washed, and challenged with LPS (0.01 μg/ml). Five hours after the addition of LPS, culture supernatants were subjected to EIA as described in Materials and Methods. Each value with a horizontal bar represents mean ± SE of four experiments. Level of significance was determined by Student's *t*-test. $*P < 0.01$ vs IFN-γ without GF109203X value. $\dagger P < 0.01$ vs Control (without IFN-γ and GF109203X) value.

LPS-dependent TNF-producibility of THP-1 cells (Fig. 3). The LPS-hyporesponsiveness of THP-1 cells induced by both h and mproTNF/3T3 cells was completely, abolished in the presence of specific anti-TNF mAb or pAbs during cocultivation (Fig. 4). Spontaneous secretion of mature TNF by hproTNF/3T3 cells was observed (about 50 to 100 units/ml by L929 cytotoxic assay, which corresponds to 25 to 50 ng/ml), however, it is likely that hyporesponsiveness of THP-1 cells against LPS responds to the contact with proTNF for the following two reasons: (1) although mproTNF/3T3 did not secrete a detectable amount of mature TNF into the culture supernatant, its desensitizing effect on the LPS-responsiveness of THP-1 cells was similar to that of hproTNF/3T3 cells (Fig. 3); (2) we previously found that mature TNF itself primes THP-1 cells.[10]

The molecular mechanism of the bidirectional role of proTNF as a single type of cytokine on a single type of target cell (THP-1) is difficult to interpret at present. One possibility is that some signal transduction pathways in THP-1 cells might differentially participate with the effects of proTNF. Biological activities of the stimuli we previously employed as priming agents (rIFN-γ, rTNF-α, and PMA) were believed to be mediated primarily through the activation of protein kinase C.[22–24] In our study, induction of the primed stage of THP-1 cells by these agents was inhibited by H7 (Fig. 6A, B), which preferentially inhibits protein kinase C as well as cyclic nucleotide-dependent protein kinases.[25] In contrast, induction of LPS-hyporesponsiveness of THP-1 cells by the proTNF/3T3 cells could not be canceled by the presence of H7 (Fig. 7), suggesting the lack of a H7-sensitive protein kinase pathway in THP-1 cells during cocultivation. This kind of H7-sensitive protein kinase is likely to be protein kinase C, because augmentation of LPS-responsiveness induced by IFN-γ was specifically abolished by GF109203X, a selective inhibitor for protein kinase C (Fig. 8).[26] TNF precursor from outside may thus induce THP-1 cells in either hyper- or hypo-responsive states to LPS for TNF production depending on the difference of coinduced signal transduction pathway.

In this regard, the observation that two different types of TNF receptor (TNF-RI: p55, and TNF-RII: p80) mediate distinct cellular responses[30] is worthy of consideration. Recently, simultaneous and efficient binding of 26-kDa membrane-bound proTNF but not 17-kDa to both types of TNF-Rs was reported leading to differences in the biological activity between proTNF and secretory TNF.[31] Furthermore, Tannenbaum *et al.*[32] reported that LPS upregulates the expression of TNF-RII mRNA, whereas IFN-γ downregulates the steady-state or LPS-induced expression of TNF-RII mRNA in murine peritoneal macrophages without any change of TNF-RI mRNA expression. Therefore, the opposite effects of proTNF on LPS-responsiveness of THP-1 cells may be mediated by the different types of TNF-Rs, which might differentially participate with protein kinase C (Ref. 33, for a review), and the balance of two different TNF-Rs may be important in determining the direction of LPS responsiveness of THP-1 cells regulated by membrane-bound proTNF.

At least 10 members of mammalian protein kinase C subdivided into three groups (cPKC; α, βI, βII, and γ: nPKC; δ, ε, η, θ: aPKC; ζ, λ; respectively) have been identified and these may be differentially activated in response to a variety of stimuli in addition to the different tissue or cellular distribution.[34] Although we did not determine the molecular species of the protein kinase C that participate in the proTNF-mediated bidirectional regulation of LPS-responsiveness of THP-1 cells, it will be necessary to investigate this matter to solve the complex regulatory roles of membrane-bound proTNF in self-reproduction.

Hyporesponsiveness of macrophage against LPS-stimulation after sequential challenge of LPS has been reported.[18–21] We separately confirmed that the level of mature TNF-production (data not shown) and the level of mRNA expression (Fig. 5) by THP-1 cells in response to secondary LPS challenge were decreased after pretreatment with primary LPS. The production

of IL-1β, another LPS-inducible cytokine, could also be suppressed by sequential challenges with LPS.[21] Although the molecular mechanisms of these processes are not yet fully understood, it was suggested that the suppression of either TNF or IL-1β production is mediated by an independent pathway because: (1) pre-exposure of macrophage to low doses of LPS inhibited the expression of TNF mRNA but did not inhibit IL-1β mRNA[20] in response to the secondary challenge of LPS; and (2) production of either TNF or IL-1β was differentially inhibited by synthetic lipids that are structurally similar to LPS.[21] In THP-1 cells, LPS-dependent expression of IL-1β mRNA was not changed, whereas expression of TNF mRNA was decreased after cocultivation with proTNF/3T3 cells (Fig. 5). Therefore, LPS-hyporesponsiveness of THP-1 cells induced by proTNF seems to be well correlated with the LPS refractory of macrophage. Our results to date thus may explain the molecular mechanism of the suppression of TNF-production at the LPS refractory stage in terms of the pro TNF-mediated autoregulatory process.

Significance of the suppressive effect of proTNF on TNF-production is unclear. However, based on its similarity to the LPS refractory state, a similar autoregulatory system might be involved in an attenuation of inflammation at the cellular level against continuous or sequential exposure with inflammatory stimuli. It can then be speculated that one biological significance of positive and negative regulations of LPS-dependent TNF-production by its precursor may be a preventive effect on an excessive biological response induced by TNF itself at the cellular level.

Modulation of TNF-producibility of macrophage might be considered representative of differentiation of this cell type. In other words, proTNF might positively or negatively regulate the ability of differentiation of THP-1 cells. Although no direct evidence of the biological role of proTNF on differentiation of embryonic cells has been reported, we found that proTNF accelerates the spontaneous differentiation of murine embryonic stem cells.[35] Kawase *et al*. also reported that mature TNF maintained the undifferentiated state of murine primordial germ cells in vitro,[36] although they did not examine the same phenomenon using proTNF. Therefore, proTNF may also regulate qualitatively the differentiation of embryonic cells depending on the target cells through bidirectional feedback regulation of its production in the inflammatory status during embryogenesis.

Our finding is quite unique in that we have proved that a single type of molecule (proTNF) can act in two ways on the same type of target cell (THP-1). Unlike mature TNF, proTNF on the cell surface might be an essential property to elicit the proper biological response of cells depending on the circumstances. These bidirectional functions of proTNF would, therefore, be useful in responding to various states in the physiological processes.

MATERIALS AND METHODS

Cell lines

Human monocyte-like leukaemia cell line THP-1[37] and murine fibroblast cell line NIH 3T3[38] were obtained from the Japanese Cancer Resources Bank (Tokyo, Japan). THP-1 cells were grown in RPMI1640 medium (Nikken Biomedical Laboratory, Kyoto, Japan) containing 10% heat-inactivated fetal calf serum (FCS, Gibco-BRL Life Technologies, Inc. Gaithersburg, MD) at 37°C in 5% CO_2–95% air. NIH3T3 cells were grown in DMEM (Nikken Biomedical Laboratory) containing 10% heat-inactivated newborn calf serum (NCS, HyClone Laboratories, Inc. Logan, UT) at 37°C in 5% CO_2–95% air and passaged on gelatin-coated plastic Petri dishes.

Reagents

The following materials were purchased from commercial suppliers: Lipofectin reagent, Geneticin (G-418 sulfate), Immunoprecipitin (fixed *Staphylococcus aureus* cells), streptoavidin–alkaline phosphatase conjugate, and ELISA amplification system (Gibco-BRL Life Technologies, Inc. Gaithersburg, MD); LPS from *Escherichia coli* 0127:B8 (Difco Laboratories, Detroit, MI); 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) (Seikagaku Corporation, Tokyo, Japan); 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF109203X) (Calbiochem-Novabiochem Co., La Jolla, CA); D-Biotinoyl-ε-aminocaproic acid *N*-hydroxy succinimide ester (biotin-CNHS-ester) (Boehringer Mannheim, Mannheim, Germany); pMC1neo-polyA (Stratagene Cloning Systems, La Jolla, CA); and [α-^{32}P]dCTP (ICN Radiochemicals, Irvine, CA); streptoavidin–biotinylated horseradish peroxidase complex (Amersham Japan, Tokyo). Enzymes used in the standard recombinant DNA techniques were purchased from several commercial suppliers without prescription. Human recombinant IFN-γ was generously provided by Toray Industries (Tokyo, Japan). Mammalian expression vector pMT2[13] was the kind gift of Dr R. J. Kaufman of the Genetics Institute (Cambridge, MA). Mouse TNF cDNA clone (pJT-1) was generously given by Dr S. Natori of Tokyo University.

Antibodies

Anti-human rTNF-α neutralizing mAb (F5H12; mouse IgG_1) and pre-immune mouse IgG were described by Tanabe *et al*.[10] Antisera for human and mouse rTNF-α were produced by immunizing rabbits and were purified according to the method of Nagai *et al*.[39] Pre-immune rabbit IgG was purchased from Endogen (Boston, MA).

Oligonucleotides

The following oligonucleotides were prepared by a model 391 DNA synthesizer (Applied Biosystems, CA) Table 1).

TABLE 1.

ATG/NcoS (sense):	5′-GACACCATG**G**GCACTGAAAG-3′
IL-1β-S (sense):	5′-CATGGCAGAAGTACCTGAGC-3′
IL-1β-A (antisense):	5′-ACCTACGCCTGGTTTTCCAG-3′
XβG5UTRS (sense):	5′-CGTCGACTCGAGCTTGTTCTTTTTGCAGAAGCTCAG AATAAACGCTCTCAACTTTGGCAGATCTACCATGGC-3′
XβG5UTRA (antisense):	5′-TCGAGCCATGGTAGATCTGCCAAAGTTGAGCGTTTA TTCTGAGCTTCTGCAAAAAGAACAAGCTCGAGTCGACGGTAC-3′

Position in bold in the sequences of ATG/NcoS indicates the sequence alteration to create the *Nco*I recognition site.

ProTNF expression plasmids

A point mutation (A → G) was introduced just downstream of the initiator methionine codon of human TNF cDNA to create the *Nco*I recognition sequence by polymerase chain reaction (PCR)-mediated mutagenesis using both ATG/NcoS primer and pBluescript T3 primer. pβGKX, as a donor plasmid of 5′-untranslated region (UTR) of Xenopus β-globin mRNA[40] to an amplified cDNA fragment lacking 5′-UTR, was constructed by insertion of a *Kpn*I–*Xho*I linker derived from complementary oligonucleotides XβG5UTRS and XβG5UTRA into the multicloning site of pBluescriptIISK(−) (Stratagene). The modified human proTNF cDNA fragment described earlier was digested by both *Nco*I and *Eco*RI, gel purified, and inserted between *Nco*I and *Eco*RI sites of pβGKX. The reulting plasmid, pβG/hu-proTNF, was digested with both *Sal*I and *Eco*RI, and subcloned into pUC18 to add the Pst I site at the 5′-end. *Pst*I–*Eco*RI fragment of this clone was then gel purified and ligated with *Pst*I–*Eco*RI digested pMT2 expression vector.[13] The resulting construct was referred to as pH-proTNF. To construct a plasmid for the expression of mouse proTNF, *Eco*RI fragment of mouse TNF cDNA (from pJT-1) was ligated into *Eco*RI-digested pMT2. Orientation of the insert was confirmed by restriction mapping and referred to as pM-proTNF.

Establishment of proTNF/3T3 cells

Ten micrograms of linearized pMT2-proTNF cDNA constructs and 1 μg of linearized pMC1neopA[15] were cotransfected to NIH3T3 cells using Lipofectin reagent according to the manufacturer's instructions. Twenty-four hours post-transfection, the medium was replaced by a fresh one containing Geneticin at 800 μg/ml. Medium was changed every 3 days until drug-resistant colonies appeared, the colonies were then pooled and maintained in medium containing 200 μg/ml of Geneticin.

Confirmation of membrane-bound proTNF expression in proTNF/3T3 cells

NIH3T3 transfectants were washed twice with PBS(−), then lysed in NP-40 lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% SDS, 1% nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin). Cell extract equivalent to 50 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions, the size-fractionated proteins were electroblotted to nitrocellulose membrane (BioTraceNT, Gelman Science, USA), then probed with anti-human or anti-mouse TNF polyclonal antibodies (pAb). Immunoreactive bands were visualized by ECL Western blotting detection system (Amersham Japan, Tokyo). To confirm whether the proTNF was actually expressed on the surface of membrane, a procedure for biotinylation of cell-surface proteins followed by immunoprecipitation using specific antibody described by Meier *et al.*[16] was carried out as follows. The adherent transfectants grown on 60-mm dishes were washed with 10 mM sodium borate, pH 8.8, 150 mM NaCl and incubated with the same buffer containing 50 μg/ml of biotin–CNHS-ester for 15 min at room temperature. The reaction was terminated by addition of NH_4Cl to 20 mM, washed extensively, then lysed as described above. Each cell lysate containing 150 μg of protein was subjected to immunoprecipitation using either anti-human TNF pAb (5 μg), anti-mouse TNF pAb (0.2 μg), or pre-immune rabbit IgG (5 μg), respectively. The procedure for immunoprecipitation was described in a previous publication.[10] Immunoprecipitated materials were size fractionated through SDS-PAGE and transferred to nitrocellulose membrane as described above. The biotinylated and immunoprecipitated proteins were then probed with streptoavidin–biotinylated horseradish peroxidase complex and visualized by ECL-Western detection system (Amersham Japan, Tokyo).

Cocultivation of THP-1 and proTNF/3T3 cells

One day before cocultivation, various NIH3T3 transfectants were trypsinized and seeded onto 60-mm gelatin-coated plastic Petri dishes at a density of 1.5×10^6 cells/5 ml without Geneticin. A set of confluent monolayers of each transfectant was washed twice with PBS(−) prior to use. Then, 5×10^6 of THP-1 cells suspended in 10% FCS/RPMI1640 were seeded onto the 3T3 feeder layer at a density of 1×10^6/ml and allowed to contact the feeder layer by natural submergence. Alternatively, THP-1 cells were pretreated with rIFN-γ at 10^3 units/ml to confirm our previous result[10] concerning the priming effect of IFN-γ through an induction of membrane-proTNF of THP-1 cells. In experiments to examine the possible involvement of protein kinase(s) in the role of membrane-bound proTNF, protein kinase inhibitors, i.e. H7 and GF109203X, were added at the stage of cocultivation or IFN-γ-pretreatment at the concentrations indicated in Figures 6–8. After 24 h, THP-1 cells were recovered, washed three times by centrifugation and challenged with LPS for 5 h as described.[10]

TNF-immunoassay

"In house" radioimmunoassay (RIA) to quantify the human TNF produced by THP-1 cells was performed as

reported.[10] During the progression of this study, an "in house" enzyme immunoassay (EIA) was developed as follows based on the same principle as RIA. A plastic 96-well multiwell plate was coated with 1 μg/ml of F5H12 anti-human TNF mAb[10] in Tris-buffered saline (TBS) for at least 5 h. The plate was washed four times with TBS containing 0.1% Tween-20 (washing buffer) and blocked with TBS containing 10% NCS. Appropriately diluted supernatants of THP-1 cells and serially diluted standard TNF-α were then added in duplicate to corresponding wells and incubated for 12 h at 4°C. The plate was washed four times with washing buffer, incubated with biotinylated anti-human TNF mAb F5H12 in TBS at 1 μg/ml for 2 h at room temperature, again washed four times with washing buffer and then incubated with 1000 × diluted streptoavidin–biotinylated alkaline phosphatase conjugate (Gibco-BRL Life Technologies, Inc.) in TBS for 2 h at room temperature. For colorimetric EIA, the ELISA amplification kit (Gibco-BRL Life Technologies, Inc.) was applied following the manufacturer's recommendation.

Statistical analysis

Statistical analyses were done by Student's *t*-test and Tukey's multiple comparison test after one-way analysis of variance. Differences were considered statistically significant if $P < 0.05$.

TNF bioassay

Cytotoxic activity of TNF was measured by L929 cytotoxic assay according to the method of Ruff and Gifford[41] with minor modification.[42]

RNA analysis

The procedure for Northern blot analysis and DNA probes to detect either human TNF mRNA or β-actin mRNA has been described.[10] cDNA probe to detect IL-1β mRNA was generated by reverse transcription/polymerase chain reaction (RT-PCR) procedure as described[43] from RNA of human placental tissue. Oligonucleotide primers used for this purpose were IL-1β-S and IL-1β-A [44] as a sense and an antisense primer, respectively. Amplified IL-1β cDNA fragment was gel purified and used for the labelling reaction. Human TNF, IL-1β, and β-actin probes were labelled with ^{32}P using a Megaprime labelling kit (Amersham Japan, Tokyo).

Acknowledgements

We thank Drs Randall J. Kaufman and Shunji Natori for providing us with the mammalian expression vector pMT2, and mouse TNF cDNA pJT-1, respectively. We also thank Drs Takashi Nishizawa and Yasuko Takeda for their critical reading and correction of the manuscript. This work was partly supported by a grant from the Kanagawa Academy of Science and Technology Foundation (KAST) of 1993.

REFERENCES

1. Sheenan KCF, Schreiber RD (1992) The synergy and antagonism of interferon-γ and TNF. In Beutler B (ed), Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine. Raven Press, New York, pp. 145–178.

2. Mizuno D (1992) Significance of Endogenous Production of TNF. In Osawa T, Bonavida B (eds), Tumor Necrosis Factor: Structure-Function Relationship and Clinical Application. Karger, Basel, pp. 1–24.

3. Tovey MG, Content J, Gresser I, Gugenheim J, Blanchard B, Guymarho J, Poupart P, Gigou M, Shaw A, Fiers W (1988) Genes for IFN-β-2 (IL-6), tumor necrosis factor, and IL-1 are expressed at high levels in the organs of normal individuals. J Immunol 141:3106–3110.

4. Hunt JS, Chen H-L. Hu X-L, Chen T-Y, Morrison DC (1992) Tumor necrosis factor-α gene expression in the tissues of normal mice. Cytokine 4:340–346.

5. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B (1975) An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 72:3666–3670.

6. Satoh M, Shimada Y, Inagawa H, Minagawa H, Kajikawa T, Oshima H, Abe S, Yamazaki M, Mizuno D (1986) Priming effect of interferons and interleukin 2 on endogenous production of tumor necrosis factor in mice. Jpn J Cancer Res 77:342–344.

7. Kitahara-Tanabe N, Tanabe Y, Morikawa A, Mizuno D, Soma GI (1991) Post-translational processing of tumor necrosis factor production. Chem Pharm Bull (Tokyo) 38:417–420.

8. Osawa T, Natori S (1989) Expression of tumor necrosis factor at a specific developmental stage of mouse embryos. Dev Biol 135:459–461.

9. Old LJ (1987) Tumor necrosis factor: polypeptide mediator network. Nature (London) 326:330–331.

10. Tanabe Y, Kitahara-Tanabe N, Mizuno D, Soma GI (1994) Enhanced production of tumour necrosis factor α (TNF-α) by its precursor on the cell surface of primed THP-1 cells. Cytokine 6:337–348.

11. Kriegler M, Perez C, DeFay K, Albert I, Lu SD (1988) A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramification for the complex physiology of TNF. Cell 53:45–53.

12. Perez C, Albert I, DeFay K, Zachariades N, Gooding L, Kriegler M (1990) A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. Cell 63:251–258.

13. Kaufman RJ, Davies MV, Pathak V, Hershey JWB (1989) The phosphorylation state of eukaryotic initiation factor 2 alters translational efficiency of specific mRNAs. Mol Cell Biol 9:946–958.

14. Shaw G, Kamen R (1986) A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective RNA degradation. Cell 46:659–667.

15. Thomas KR, Cappecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryonic stem cells. Cell 51:503–512.

16. Meiyer T, Arni S, Mlarkannan S, Poincelet M, Hoessli D (1992) Immunodetection of biotinylated lymphocyte-surface proteins by enhanced chemiluminescence: a nonradioactive method for cell-surface protein analysis. Anal Biochem 204:220–226.

17. Nii A, Sone S, Orino E, Ogura T (1993) Induction of a 26-kDa membrane-form tumor necrosis factor (TNF) in human alveolar macrophages. J Leukocyte Biol 53:29–36.

18. Zuckerman SH, Evans GF, Snyder YM, Roeder WD (1989) Endotoxin-macrophage interaction: Post-translational regulation of tumor necrosis factor expression. J Immunol 143:1223–1227.

19. Mathison JC, Virca GD, Wolfson E, Tobias PS, Glaser K, Ulevitch RJ (1990) Adaptation to bacterial lipopolysaccharide control lipopolysaccharide-induced tumor necrosis factor production in rabbit macrophages. J Clin Invest 85:1108–1118.

20. Takasuka N, Tokunaga T, Akagawa KS (1991) Preexposure of macrophages to low doses of lipopolysaccharide inhibit the

expression of tumor necrosis factor-α mRNA but not of IL-1β mRNA. J Immunol 146:3824–3830.

21. Fahmi H, Chaby R (1993) Desensitization of macrophages to endotoxin effects is not correlated with a down-regulation of lipopolysaccharide-binding site. Cell Immunol 150:219–229.

22. Gumina RJ, Freire-Moar J, de Young L, Webb DR, Devens BH (1991) Transduction of the IFN-γ signal for HLA-DR expression in the promonocytic line THP-1 involves a late-acting PKC activity. Cell Immunol 138:265–279.

23. Schutze S, Nottrott S, Pfizenmaier K, Kronke M (1990) Tumor necrosis factor signal transduction; cell-type-specific activation and translocation of protein kinase C. J Immunol 144:2604–2608.

24. Aihara H, Asaoka Y, Yoshida K, Nishizuka Y (1991) Sustained activation of protein kinase C is essential to HL-60 cell differentiation to macrophage. Proc Natl Acad Sci USA 99:11062–11066.

25. Kawamoto S, Hidaka H (1984) 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochem Biophys Res Commun 125:258–264.

26. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J (1991) The bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15781.

27. Yamasu K, Onoe H, Soma GI, Oshima H, Mizuno D (1989) Secretion of tumor necrosis factor during fetal and neonatal development of the mouse: Ontogenic inflammation. J Biol Response Mod 8:644–655.

28. Kohchi C, Noguchi K, Tanabe Y, Mizuno D, Soma GI (1994) Constititive expression of TNF-α and -β genes in mouse embryo: Roles of cytokines as regulator and effector on development. Int J Biochem 26:111–119.

29. Inagawa H, Oshima H, Soma GI, Mizuno D (1988) TNF induces endogenous TNF in vivo: The basis of EET therapy as a combination of rTNF together with endogenous TNF. J Biol Response Mod 7:596–607.

30. Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino Jr. MA, Goeddel DV (1991) The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc Natl Acad Sci USA 88:9292–9296.

31. Grell M, Douni E, Wajant H, Löhden M, Clauss M, Maxeiner B, Georgopoulos S, Lesslauer W, Kollias G, Pfizenmaier K, Scheurich P (1995) The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell 83:793–802.

32. Tannenbaum CS, Major JA, Hamilton TA (1993) IFN-γ and lipopolysaccharide differentially modulate expression of tumor necrosis factor receptor mRNA in murine peritoneal macrophages. J Immunol 151:6833–6839.

33. Aggarwal BB, Katarajan K (1996) Tumor necrosis factors: Developments during the last decade. Eur Cytokine Netw 7:93–124.

34. Mahoney CW, Huang KP (1994) Molecular and catalitic properties of protein kinase C. In Kuo JF (ed) Protein Kinase C, Oxford University Press, New York, pp. 16–63.

35. Kohchi C, Tanabe Y, Noguchi K, Mizuno D, Soma GI (1996) Induction of differentiation in embryonic stem cells by 26-kD membrane-bound tumor necrosis factor (TNF) and 17-kD free TNF. In Vivo 10:19–28.

36. Kawase E, Yamamoto H, Hashimoto K, Nakatsuji N (1994) Tumor necrosis factor-α (TNF-α) stimulates proliferation of mouse primordial germ cells in culture. Dev Biol 161:91–95.

37. Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, Konno T, Tada K (1982) Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Cancer Res 42:1530–1536.

38. Jainchill JL, Aaronson SA, Todaro GJ (1969) Murine sarcoma and leukemia viruses: Assay using clonal lines of contact-inhibited mouse cells. J Virol 4:549–553.

39. Nagai M, Saigusa T, Shimada Y, Inagawa H, Oshima H, Iriki M (1988) Antibody to tumor necrosis factor (TNF) reduces endotoxin fever. Experientia (Basel) 44:606–607.

40. Falcone D, Andrews DW (1991) Both the 5′ untranslated region and the sequences surrounding the start site contribute to efficient initiation of translation in vitro. Mol Cell Biol 11:2656–2664.

41. Ruff MR, Gifford GE (1980) Purification and physicochemical characterization of rabbit tumor necrosis factor. J Immunol 125:1671–1677.

42. Gatanaga T, Takahashi K, Yamazaki M, Mizuno D, Abe S (1985) Combination antitumor therapy with rabbit tumor necrosis factor and chemo- and immuno-therapeutic agents against murine tumors. Jpn J Cancer Res 76:631–636.

43. Kohchi C, Mizuno D, Soma GI (1991) Expression of tumor necrosis factor-α and -β transcripts in embryonal carcinoma and trophoblast cell lines: inflammation-like state as possible regulatory mechanism for ontogenesis. Eur Cytokine Netw 2:245–255.

44. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price VL, Gillis S, Henney CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP, Cosman D (1985) Cloning, Sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature (London) 315:641–647.