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Secretions of MMP-9 By Soluble Glucocorticoid-Induced Tumor Necrosis Factor Receptor (sGITR) Mediated By Protein Kinase C (PKC)δ and Phospholipase D (PLD) in Murine Macrophage

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Abstract The secretion of matrix metalloproteinase (MMP-9) is stimulated by the glucocorticoid-induced tumor necrosis factor receptor (GITR), a new tumor necrosis factor receptor (TNFR) family, in murine macrophages via an activation of protein kinase C (PKC)δ and phospholipase D (PLD). Secretions of MMP-9 are stimulated by the phosphatidic acid (PA), a product of PLD activity and an inhibition of PA production by a 1-propanol inhibited secretion of MMP-9 by soluble GITR (sGITR). MMP-9 is not secreted by diacylglycerol (DAG) and an inhibitor of PA phosphatase has no effect on the secretion induced by sGITR, indicating that PA is responsible for MMP-9 secretion in murine macrophages. Our data indicates that sGITR-induced activation of PKCδ and PLD increases MMP-9 secretions in macrophages. J. Cell. Biochem. 92: 481–490, 2004. © 2004 Wiley-Liss, Inc.

Key words: GITR; MMP-9; macrophages; PKCδ; PLD

The glucocorticoid-induced tumor necrosis factor receptor (TNFR) family-related gene (GITR), a 66–70 kDa homodimeric glycoprotein, is a member of the tumor necrosis factor-nerve growth factor (TNF-NGF) receptor family and was exclusively induced in T-cells by dexamethasone [Nocentini et al., 1997]. Although, no homology was found in the intracellular domain among TNFR members, GITR shares homologies for the intracellular domain with 4-1BB, CD27, and TR11, indicating that those subfamilies may share signal transduction pathways [Kwon and Weismann, 1989; Arch and Thompson, 1998; Kwon et al., 1999]. Recently, it has been demonstrated by Shimizu et al. [2002] that GITR is predominantly expressed on $CD25^+CD4^+$ regulatory T-cells in the thymus and its periphery, and its monoclonal antibodies abrogate $CD25^+CD4^+$ T-cell-mediated suppression in vitro. In addition, an increased expression of GITR in $CD25^+CD4^+$ T-cells has also been detected by DNA microarray [Mchugh et al., 2002]. These results suggest that the function of GITR is implied to be related to the control of immunological self-tolerance. Soluble GITR (sGITR) activated macrophages and induced nitric oxide synthase [Shin et al., 2002a], cyclooxygenase-2 [Shin et al., 2002b], and matrix metalloproteinases (MMPs) [Lee et al., 2003], probably via the binding of GITR to its ligand. It has been generally considered that the TNF family transmits signals through the receptor, TNFR. However, upon binding of TNF to TNFR, signals are produced in two directions through both TNF and TNFR. Reverse signaling through 4-1BB ligand inhibited proliferation, induced apoptosis, and stimulated

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monocytes to generate IL-8 [Langstein et al., 1998]. The triggering of TNF-related apoptosis inducing ligand (TRAIL) by DR4-Fc induced maximal proliferation response and enhanced interferon (INF)-γ secretion by activated T-cells [Chou et al., 2001].

MMPs are a family of zinc-containing endoproteinases. They are a family of extracellular matrix-degrading enzymes that include the interstitial collagenases, gelatinases or type IV basement membrane collagenases, stromelysins, matrilysin, metalloelastase, and membrane-type MMPs [Birkedal-Hansen, 1995]. The lower level of mostly gelatinase-type MMPs produced by T-cells principally serves to facilitate T-cell migration through connective tissues, whereas the higher level of a broader range of MMPs produced by the macrophages also plays the roles in the degradation, the removal, and the remodeling of connective tissues. The production, secretion, and activation of MMPs by both immune cells are regulated by cytokines and inflammatory mediators [Nagase and Woessner, 1999]. The potential importance of the many activities of MMPs in integrated immune and inflammatory responses has been suggested by the inhibitory effects of MMP inhibitors in several animal models of human diseases [Conway et al., 1995].

In our study, sGITR-induced MMP-9 secretion was mediated via PKCδ and phospholipase D (PLD). Phosphatidic acid (PA) formation by PLD activation was involved in the secretion of MMP-9.

MATERIALS AND METHODS

Cells and Reagents

Raw264.7 cells, a murine macrophage cell line, were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin in 6-well plates or in 10-cm dishes.

Recombinant extracellular domain of GITR was produced in *E. coli* and purified as described before [Shin et al., 2002a]. SDS gel-purified sGITR contained no more than a trace amount of endotoxin (<0.4 EU/mg by E-Toxate assay from Sigma Chemical Co., St. Louis, MI). All sGITR preparations were pretreated with polymyxin B for 2 h at room temperature to prevent sGITR from endotoxin contamination. Extracellular domain of TNFRH1 was also produced in *E. coli* as a fusion protein with a polyhistidine tag and was used as a negative control. TNFRH1 was a generous gift from Dr. B. Kwon in IRC, UOU, Korea.

Immunoblot Analysis

Indicated samples were separated by SDS–PAGE in the absence of a reducing agent and transferred onto nitrocellulose membranes. Some samples were concentrated by ultrafiltration using membrane units with molecular weight limit 10,000 Da (Sigma Chemical Co.). The blots were then washed in Tris-Tween buffered saline (TTBS, 20 mM Tris-HCl, pH 7.6 containing 137 mM NaCl and 0.05% (v/v) Tween 20), blocked overnight with 5% (w/v) bovine serum albumin (BSA), and probed with polyclonal antibody (Ab) for MMP-9 and monoclonal Ab for PKCδ in 5% (w/v) BSA dissolved in TTBS. Using horse-radish peroxidase (HRP)-conjugated secondary anti-rabbit Ab (MMP-9) and anti-mouse Ab (PKCδ) were detected by enhanced chemiluminescence.

RNA Isolation and RT-PCR

Expression of MMP-9 and glyceraldehydes-3-P dehydrogenase (GAPDH) mRNA was assessed by RT-PCR analysis. RNA was isolated from sGITR-treated and control cells using TRI reagent (Sigma Chemical Co.). Total RNA was used for cDNA synthesis by reverse transcriptase of cDNA synthesis kit (Invitrogen, San Diego, CA). The cDNA were amplified using PCR for 30 cycles (MMP-9) and 25 cycles (GAPDH) with the following specific PCR primers: mouse MMP-9, 5′-CCCACATTT-GACGTCCAGAGAAGAA-3′ (forward) and 5′-GTTTTTGATGCTATTGCTGAGATCCA-3′ (reverse); mouse GAPDH, 5′-ACCACAGTC-CATGCCATCAC-3′ (forward) and 5′-TCCAC-CACCCTGTTGCTGTA-3′ (reverse). Each cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C. GAPDH was used as the internal control. The size of PCR products for mouse MMP-9 and GAPDH were 208 and 452 bp, respectively.

PKC Translocation Assay

Raw264.7 cells were seeded in 24-well plates at 1×10^6 cells/well. The cells were incubated in DMEM with 10% FBS, stimulated with 1 μg/ml of sGITR for indicated time periods. Cells were harvested and washed with ice-cold

phosphate-based saline. Then, cells were resuspended in lysis buffer A (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM EDTA, and protease inhibitor mixtures) and homogenized at 4°C through several times of pipetting. The homogenate was centrifuged at 1,000*g* for 5 min at 4°C. The supernatant was collected and centrifuged at 100,000*g* for 1 h at 4°C. The supernatant was collected as a cytosolic fraction. The pellet was resuspended in lysis buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10 mM EDTA, and protease inhibitor mixtures) and sonicated for 10 s. The suspension was collected as a membrane fraction.

PLD Assay

PLD activity was measured by the method of Williger et al. [1999]. For PLD assay, 6-well plates were seeded with 2×10^6 cells. The cells were serum-deprived and labeled in DMEM, 0.1% BSA with 1 μCi/ml [^{3}H]myristic acid for 18 h. The serum-deprived and labeled cells were washed with DMEM, 0.1% BSA for two times to remove unincorporated radioactivity. The cells were incubated in DMEM, 0.1% BSA, and 0.3% 1-butanol for 20 min and stimulated with indicated concentration of sGITR for indicated times. Cells were washed with phosphate-based saline and 0.1% BSA, scraped with 1 ml of ice-cold methanol, transferred into glass-tubes. Chloroform and 0.1 M HCl were added to a final ratio of 1:1:1. The lipid-containing lower phase was collected, dried under a nitrogen stream, and dissolved in 30 μl of methanol:chloroform (1:1). An aliquot was taken to measure radioactivity of the lipids. Phosphatidylbutanol (PtdBut) was separated by thin-layer chromatography using Whatman LK6D silica gel plates and a solvent system (ethyl acetate:isooctane: acetic acid:water, 110:50:20:100). Tritiated PtdBut was measured after visualizing authentic standard (Avanti Polar lipids, Inc.) with iodine vapor.

RESULTS

Soluble GITR-Induced MMP-9 Mediated Via Activation of PKCδ

In our initial studies [Lee et al., 2003], an extra-cellular domain of GITR activated murine macrophages to express matrix metalloproteinase-9 (MMP-9) in dose- and time-dependent manners. GITR and GITR ligand were constitutively expressed on the surface of the Raw264.7 macrophage cell line [Shin et al., 2002a]. To investigate the signal transduction pathway to secrete MMP-9, we tested the effects of the inhibitors of MEK (PD98059), p38 (SB203580), tyrosine kinase (genistein), H-89 (PKA), phosphatidyl inositol (PI)-3 kinase (LY294002), and NF-κB (pyrrolidinedithiocarbamate (PDTC)) on sGITR-mediated MMP-9 secretion (data not shown). Soluble GITR-induced MMP-9 secretion was not inhibited by the addition of these inhibitors, suggesting that these molecules had little chance to be involved in sGITR-induced MMP-9 secretion. However, rottlerlin, a PKCδ-specific inhibitor, blocked sGITR-induced MMP-9 secretion, whereas Ro-31-8425, a PKCδ inhibitor, did not (Fig. 1A). This result indicated that PKCδ was involved in sGITR-stimulated MMP-9 secretion among various PKC isoforms. In addition, TNFRH1 as a negative control did not induce MMP-9 secretion (Fig. 1B).

Since the translocation of PKC to a particulate fraction is the key step for the activation of this enzyme [Pongracz et al., 1999], determination of PKC content in cellular membranes reflects PKC activity. To investigate whether PKCδ was activated by sGITR stimulation, we measured the distribution of PKCδ in the membrane and cytosol fraction by Western blot using PKCδ-specific Ab. PKCδ activity started to increase 2 h after sGITR stimulation and maximum activity of PKCδ was detected at 4 h in the membrane fractions of Raw264.7 cells stimulated by sGITR (Fig. 1C). On the other hand, TNFRH1, which did not induce MMP-9 secretion, did not show PKCδ translocation. These data strongly suggested that sGITR-evoked activation of PKCδ is responsible for MMP-9 secretion. To further characterize the signaling pathways for sGITR-induced MMP-9 secretion, we investigated the possible involvement of phospholipase C (PLC). Figure 1D showed that sGITR-induced MMP-9 secretion was not inhibited by the treatment of the PLC inhibitor U73122 indicating that PLC was not involved in sGITR-induced MMP-9 secretion.

Phopholipase D (PLD) Activation Involved in the Induction of MMP-9 Secretion Stimulated by sGITR

Since PKC isoforms have a broad spectrum of effects including the activation of PLD [Exton,

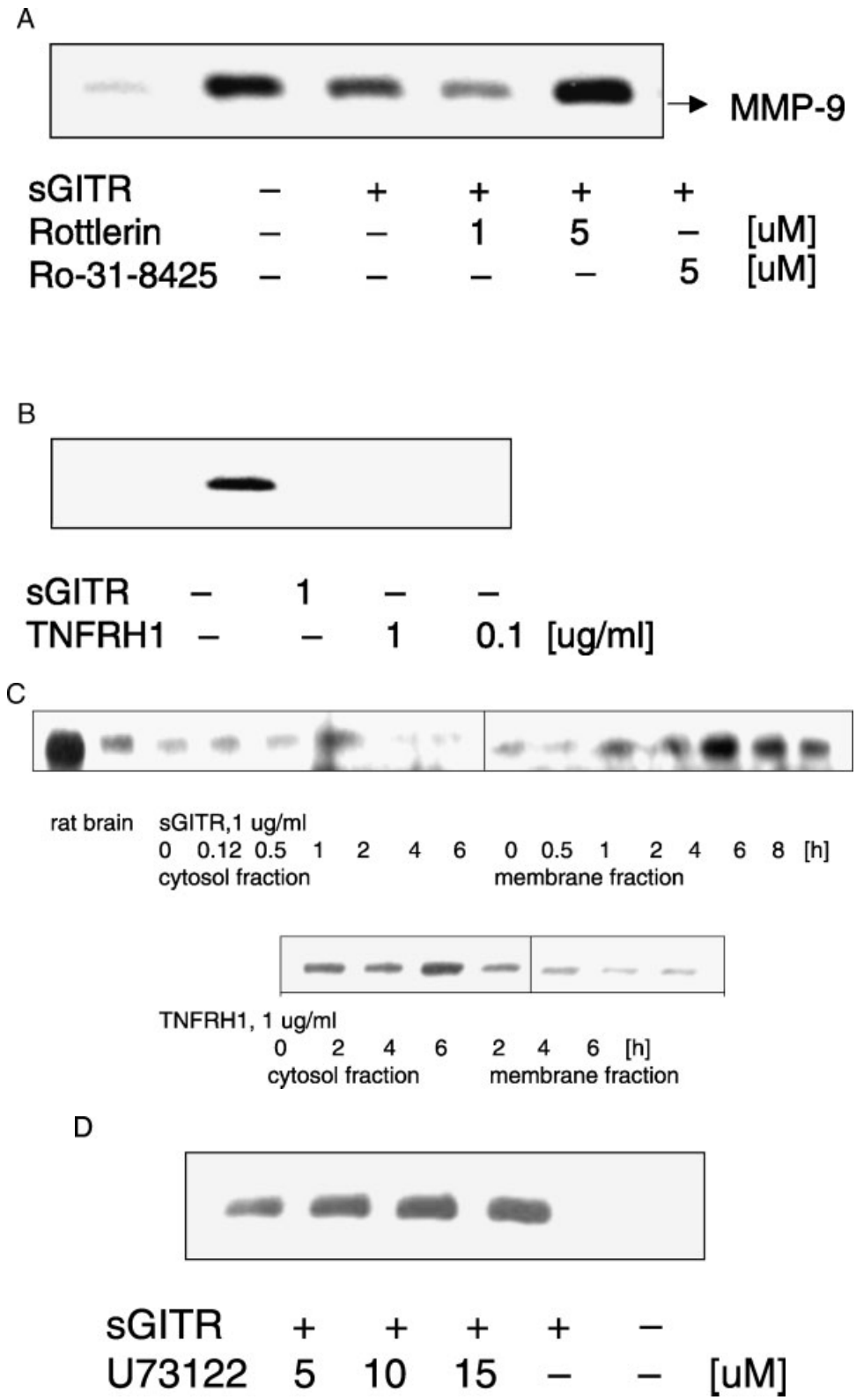

Fig. 1. Effects of sGITR on MMP-9 secretion in the absence or presence of inhibitors, and on activation of PKCδ. Cells were pretreated without or with inhibitors for 1 h, and then 1 μg/ml sGITR (**A**, **D**) or TNFRH1 (**B**) was added. After 8 h, medium samples, 10 μl each, were separated on 10% SDS–PAGE gels and were analyzed for MMP-9 by Western blot as described in Materials and Methods. Cells were stimulated with the 1 μg/ml of sGITR or TNFRH1 for the indicated times. Harvested cells were fractionated and analyzed for detection of PKCδ by Western blotting with anti-PKCδ Ab (**C**). Rat brain protein was used as a positive control. The data shown are representative of three independent experiments.

1997a], we tested the possible involvement of PLD in the stimulation of MMP-9 by sGITR. We measured if sGITR induced PLD activation in Raw264.7 cells. Raw264.7 cells labeled with [^{3}H]myristate are stimulated by addition of sGITR for various times, and the formation of PtdBut from 1-butanol was measured for PLD activity. As shown in Figure 2A, PtdBut formation was slowly induced and detectable at 30 min, reached a maximum at 2 h, and remained up to 10 h after sGITR stimulation. The response was detectable with 0.2 μg/ml sGITR and maximal at 1 μg/ml sGITR (Fig. 2B). When the cells were pretreated with rottlerin, the PLD activity induced by sGITR was decreased (Fig. 2C). On the other hand, TNFRH1 as a negative control did not affect PLD activation (Fig. 2D). These results suggested that sGITR-mediated PKCδ activation is coupled to PLD activation and that PLD is located downstream from PKCδ after sGITR stimulation.

The participation of PLD in the pathway of MMP-9 secretion was supported when PLD activity was inhibited. When 1-propanol was used to block PA production by PLD activity before sGITR stimulation, the secretion of MMP-9 was gradually inhibited by increasing the concentration of 1-propanol (Fig. 3A). As a negative control, 2-propanol pretreatment also inhibited MMP-9 secretion, but the degree was much less. The secretion of MMP-9 was also measured at the fixed concentration of 1-propanol or 2-propanol with various concentrations of sGITR stimulation to discriminate the effects of 1-propanol and 2-propanol. MMP-9 secretion was increased by increasing the concentration of sGITR in the presence of 2-propanol, but not in the presence of 1-propanol

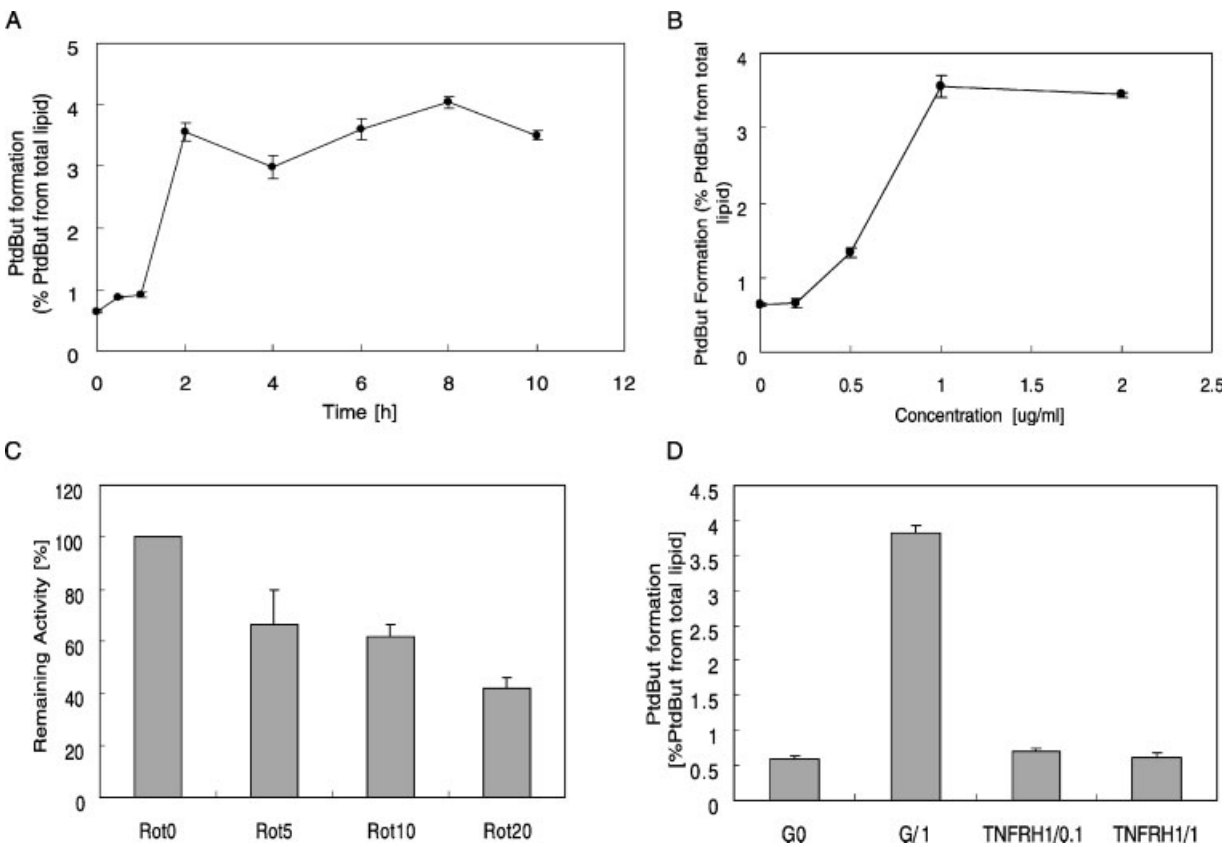

Fig. 2. Activation of PLD in sGITR-treated Raw264.7 cells. Cells, at their log phase of growth, were serum-deprived and labeled with 1 μCi/ml [^{3}H]myristic acid. Following washing and preincubation with DMEM, 0.1% BSA, 0.3% 1-butanol for 20 min, 1 μg/ml sGITR was added, and cells were incubated for the indicated times at 37°C (**A**). Or cells were stimulated with various concentrations of sGITR (**B**) in the presence or absence of rottlerin (**C**) for 2 h. Cells were treated with TNFRH1 (0.1 or 1 μg/ml) (**D**) for 2 h. Lipids were extracted and analyzed, and the production of [^{3}H]PtdBut was analyzed as described under Materials and Methods. [^{3}H]PtdBut values were normalized by dividing the measured counts/min by the counts/min in the total lipid fraction. Data are expressed as the mean ± SD of the fold activations in triplicate. Results are representative of three experiments.

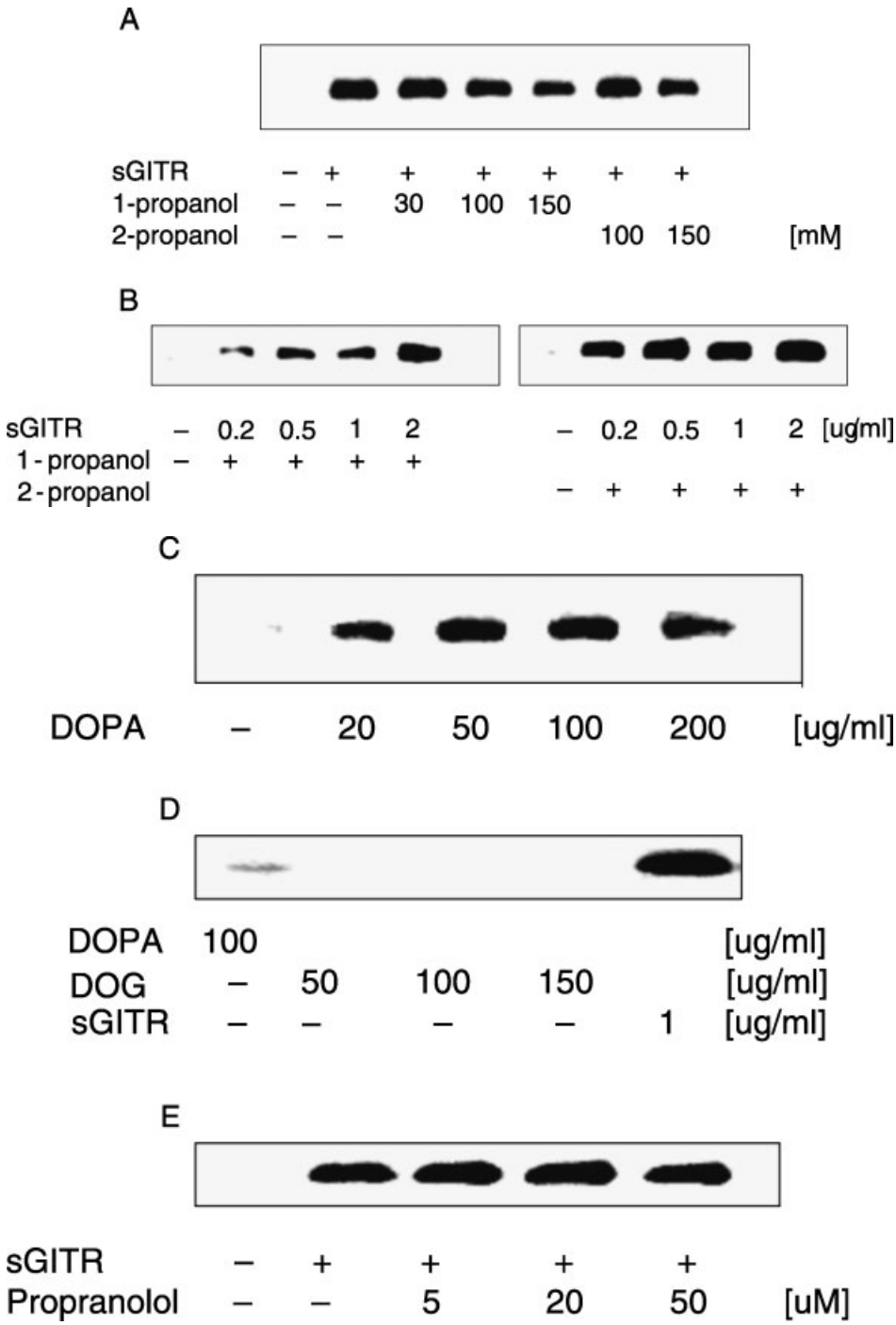

Fig. 3. Effects of sGITR without or with inhibitors or other effectors on MMP-9 secretion. Cells were pretreated with 1-propanol or 2-propanol (**A**); propranolol (**E**) for 1 h, and then 1 μg/ml sGITR was added for 8 h after which 10-μl media samples were analyzed for MMP-9 secretion by Western blot as described in Materials and Methods. Cells were incubated with 100 mM of 1-propanol or 2-propanol for 1 h, and then different concentration of sGITR was added (**B**). Cells were incubated with the indicated concentrations of DOPA (**C**) or dioctanoylglycerol (**D**) for 8 h after which 100-μl media samples were concentrated and analyzed for MMP-9 secretion. The data shown are representative of three independent experiments.

(Fig. 3B). Next, we examined whether PA or its metabolite diacylglycerol (DAG) played a role in signaling the release of MMP-9 after activation of PLD by sGITR. The cells were treated with various concentrations of a short chain PA, dioctanoylphosphatidic acid (DOPA). Figure 3C showed that DOPA induced MMP-9 secretion in a dose-dependent manner with secretion reaching a plateau at 50 μg/ml. Cells were also incubated with various concentrations of dioctanoylglycerol, and then medium samples were assayed for detection of MMP-9. Dioctanoylglycerol did not induce the release of MMP-9 even at concentrations higher than those of PA as shown in Figure 3D. In additional experiments, the secretion of MMP-9 stimulated by sGITR was examined after the cells were pretreated with propranolol, an inhibitor of PA phosphatidase. Figure 3E shows that propranolol did not lower the level of MMP-9 induced by sGITR. Our results showed that PA, not DAG, could play a role as a signaling molecule to release MMP-9 after PLD activation by sGITR stimulation.

We also investigated whether or not MMP-9 production was regulated at the transcriptional level. The mRNA levels of MMP-9 after sGITR treatment were measured by RT-PCR. As shown in Figure 4, sGITR activated the expression of MMP-9 mRNA in a time-dependent manner, indicating that treatment of cells with sGITR increased expression of MMP-9 mRNA.

Taken together, our results suggested that MMP-9 secretion by sGITR can be regulated by PKCδ and PLD activation.

DISCUSSION

We have shown that sGITR activates macrophages to secrete a high level of MMP-9 in Raw264.7 cells [Lee et al., 2003]. Both GITR and its ligand were constitutively expressed on the surface of macrophages, even at a low level [Shin et al., 2002a]. Recently, Yu et al. [2003] also demonstrated the expression of GITR ligand in Raw274.7 cells and dendritic cells. In this study, we have shown that sGITR-induced MMP-9 secretion was mediated via the activation of PKCδ and PLD.

Activation of MMP-9, the quantitatively predominant MMP of T-cells and macrophages, through its cleavage by other MMPs, is of special interest in the field of immunology. Macrophage MMPs degrade connective tissues in the course of remodeling, with resultant release of stored growth factors, and in diseases that may injure normal structures. Injection of MMP inhibitors suppressed the induction and progression of experimental autoimmune encephalomyelitis in mice with a drastic decrease in gelatinolytic activity in cerebrospinal fluid suggesting the potential importance of MMPs in integrated immune and inflammatory responses [Conway et al., 1995]. Our results also suggested that MMP-9 generated from macrophages might be responsible for tissue destruction after injecting sGITR [Lee et al., 2003].

PLD is widely distributed in mammalian cells, where it is regulated by a variety of hormones and extracellular signals. It hydrolyzes phosphatidylcholine to PA and choline [Exton, 1997a]. Although the physiological functions of PLD need to be defined, several mechanisms might be possible for the biological effects of PLD. PLD changes the lipid composition of cellular membranes by hydrolyzing phosphatidyl choline or by generating molecules which have signaling functions. One of its products, PA, may interact with several molecules which are located in membranes or intracellular compartments and whose function is related to signal transduction pathways. Although PA is known to influence Ca^{2+}-independent form of PKC by generating a metabolite of PA, DAG [Exton, 1997b], sGITR-induced MMP-9 secretion via PA was not due to DAG in our system. Wakelam et al. [1997] have also demonstrated that the PA produced by PLD caused MMP-9 synthesis and activation.

Although controversial, there is abundant evidence that PLD is regulated by PKC. Increased PLD activity has been shown to be related with the expression of the mRNA of PKC isoforms α, β, δ, ε, ζ, and θ in HL-60 cells [Siebenhener and Wooten, 1993]. An important aspect of PKC action is its synergistic interaction with PLD activating factors such as ADP ribosylation factor and RhoA to activate PLD. As demonstrated in HL-60 cells, phorbol-12-myristate 13-acetate-mediated protein phosphorylation by PKC may not be directly involved in PLD activation [Ohguchi et al., 1995]. PKC may act by phosphorylating the proteins involved in PLD regulation or by altering the response of PLD. In murine macrophages stimulated by sGITR, PKCδ was found to be located upstream from PLD since the

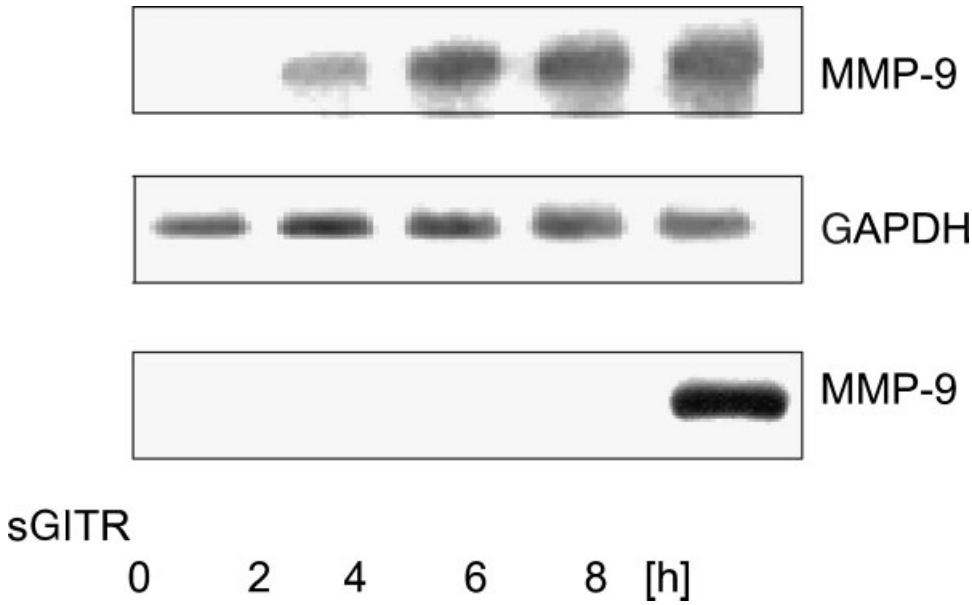

Fig. 4. Effects of sGITR on the expression of mRNA of MMP-9. Cells were cultured and stimulated by adding 1 μg/ml of sGITR for 0, 2, 4, 6, and 8 h. Total RNA was extracted and subjected to RT-PCR analysis. In the bottom lane, secreted MMP-9 level was determined by Western blot. Similar results were obtained in three independent experiments.

pretreatment of rottlerin inhibited PLD activation stimulated by sGITR. However, we do not know whether or not PLD is directly activated by PKCδ.

Although rottlerin almost completely reduced sGITR-induced MMP-9 secretion, this concentration was a 40% inhibition of PLD activity. This suggests that there could be other mechanisms besides PLD for downstream targets for PKCδ to induce MMP-9 secretion. Several laboratories have investigated the signal transduction pathways responsible for MMP-9 regulation. Their results indicate that, depending on the stimulus and cell types, induction of MMP-9 synthesis involves multiple signaling cascades including p38 kinase [Simon et al., 1998], extracellular signal-regulated kinases (ERK1/2) [Liu et al., 2002], jun-N-terminal kinase (JNK) [Gum et al., 1997], and tyrosine kinase [McCawley et al., 1999]. In MCF breast cancer cells stimulated with FGF-2, ERK1/2 activation was involved in downstream targets for PKCδ [Liu et al., 2002]. Since PD98059, SB203580, genistein did not affect sGITR-stimulated MMP-9 secretion in our system, it appears that there is little chance that ERK1/2, p38 kinase, or tyrosine kinase play a role in sGITR-stimulated MMP-9 secretion, although this is only based on pharmacological data. This either leaves an activation of JNK that we have not determined or there might be other signaling pathways in mediating sGITR-induced MMP-9 production. The 5′-flanking region of MMP-9 gene contains binding sequences for NF-κB and AP-1. Protein kinase C (PKC) is regarded as a potential inducer of AP-1 and inhibition of PKCδ affected significantly MMP-9 secretion by sGITR. It may be possible that sGITR-stimulated MMP-9 induction is, at least in part, under the control of AP-1 in macrophages.

Our data showed that sGITR-mediated activation of PKCδ/PLD and subsequent production of PA are responsible for MMP-9 secretion in murine macrophage. This study also strongly suggested that sGITR-mediated upregulation of MMP-9 mRNA cause to enhance MMP-9 secretion. However, secretion of MMP-9 could be stimulated by a number of factors and regulated at both the transcriptional and translational level. Treatment of Balb 3T3 cells with IL-1β activated MMP-9 transcription and subsequent secretion [Ruhul Amin et al., 2003]. The regulation of eosinophil-derived MMP-9 by TNF-α was regulated at both the transcriptional and translational level [Schwingshackl et al., 1999]. There are also other possible mechanisms by which sGITR increases MMP-9 secretion. Soluble GITR-mediated signaling pathways could directly enhance MMP-9 secretion, which leads

to a compensatory increase in cellular MMP-9 contents via activation of transcription of the MMP-9 gene. Alternatively, sGITR-evoked signals could simultaneously increase both MMP-9 secretion and its transcription. Several secreting proteins have been known to be released from cells by receptor-mediated signals, which evoke upregulation of their transcriptions by the mechanism of stimulus-secretion-synthesis coupling [Eiden et al., 1984].

In conclusion, the present study provides the characterization of MMP-9 secretion signaling pathways activated by sGITR in murine macrophages. The presence of GITR ligand has been shown in murine macrophage cells [Shin et al., 2002a; Yu et al., 2003]. Since both the GITR and GITR ligand were found in murine macrophages, it is not clear whether sGITR-stimulated MMP-9 induction was caused by signal transduction through the GITR ligand or by blocking the endogenous GITR signal. However, we have demonstrated that stimulation by sGITR led to an activation of PKCδ and PLD, and resulted in MMP-9 secretion. While further studies are required to delineate the signaling pathway leading to MMP-9 secretion by sGITR, this report characterizes MMP-9 secretion signaling events in response to sGITR.

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