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# Synergistic and selective stimulation of gelatinase B production in macrophages by lipopolysaccharide, *trans*-retinoic acid and CGP 41251, a protein kinase C regulator [1]

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**Abstract**

The production of gelatinase B by macrophages is relevant in the immunological and migratory functions of macrophages. CGP 41251, an inhibitor of protein kinase C (PKC), was found to stimulate the expression of gelatinase B in macrophages, as shown by the study of two different monocytic/macrophagic cell lines, mouse RAW 264.7 and human THP-1 cells. When human monocytes and rat peritoneal macrophages were treated with CGP 41251, insignificant increases of 10 and 25% were obtained. This can possibly be due to the presence of contaminating cells in these two enriched populations, since the CGP 41251 treatment of non-macrophagic cell lines inhibited their PMA-induced gelatinase B production. Taken together, these results suggest that the stimulatory effect of CGP 41251 is specific to cells of the monocytic lineage. Using RAW 264.7 cells as a model, the effect of CGP 41251 is additive to that obtained using lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA), as revealed by gelatin zymography and Northern blot analysis. The stimulatory effect of CGP 41251 on gelatinase B production in RAW 264.7 was: (a) inhibited by calphostin C (as is the LPS-induced response), indicating a PKC-dependence; (b) inhibited by dexamethasone (as opposed to the LPS-induced response); and (c) enhanced by addition of *trans*-retinoic acid (RA). In fact, RA can induce gelatinase B production, either alone or in synergy with LPS and/or CGP 41251, since the combination of the three agents gives the highest gelatinase B response, at both the protein and the mRNA levels. This represents an important observation considering that RA is now being tested as an anti-cancer agent and proposed for prevention studies.

*Keywords:* Gelatinase B; Protein kinase C; Lipopolysaccharide; *trans*-Retinoic acid

## 1. Introduction

Chronic inflammatory lesions, such as periodontitis, rheumatoid arthritis or interstitial nephritis, are infiltrated with large numbers of mononuclear leukocytes [1,2]. Among these cells, macrophages are now recognized as major effectors in the degradation and the turnover of extracellular matrices (ECM). Macrophages can regulate interstitial and synovial fibroblast functions through the secretion of several cytokines, particularly TNF-$\alpha$ and IL-1 [3]. Furthermore, macrophages can also be fine-tuned for controlled proteolysis of connective tissues through the release of matrix metalloproteinases (MMPs) and other

Abbreviations: **ECM**, extracellular matrices; **FBS**, fetal bovine serum; **LPS**, lipopolysaccharide; **MMP**, matrix metalloproteinase; **NO**, nitric oxide; **PKC**, protein kinase C; **PMA**, phorbol 12-myristate 13-acetate; **RA**, *trans*-retinoic acid.

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ECM-remodelling enzymes [4,5]. Recently, gelatinase B (MMP-9; EC 3.4.24.35), an enzyme known to cleave different types of collagen, gelatin and some other substrates (reviewed in Refs. [6–8]), received much attention. Gelatinase B is a 92 kDa enzyme secreted by a number of normal and transformed cells. Neutrophils [9], T-lymphocytes [10,11] and monocytes/macrophages [4,12] were all reported to produce gelatinase B. In monocytes and in monocytic cell lines, constitutive levels of gelatinase B appear to be rather low; however, these levels are inducible, at both mRNA and protein levels, by agents such as lipopolysaccharide (LPS), concanavalin A and phorbol 12-myristate 13-acetate (PMA) [13,14]. This activation seems to follow a prostaglandin $E_2$/cAMP-dependent pathway and can be down-regulated by IL-4 and interferon gamma [1,15]. Using U937, a human monocytic-like cell line, Saarialho-Kere et al. [16] reported that the induction of gelatinase B production by LPS occurs at a pretranslational level. Furthermore, it was suggested that gelatinase B production by U937 cells was regulated by a mechanism independent of the differentiation to macrophage-like cells [17].

The murine RAW 264.7 cell line is a macrophage cell line originally established from the ascites of a tumor induced in a BAB/14 mouse by the intraperitoneal injection of Abelson leukemia virus [18]. Numerous studies have utilized the RAW 264.7 cell line as a macrophage model, particularly for the study of LPS stimulation [19], either at the level of TNF-$\alpha$ [20] or nitric oxide [21]. This extensive use of RAW 264.7 has now established this cell line as a prototype for the study of macrophage functions. Using the RAW 264.7 cell line, our group recently observed contrasting effects of two protein kinase C (PKC) inhibitors, calphostin C and staurosporine, on both LPS- and PMA-induced gelatinase B production [22]. Calphostin C completely abolished gelatinase B production, whereas staurosporine enhanced it 3- to 4-fold. When tested alone, doses of staurosporine lower than 100 nM had no effect on gelatinase B production while doses higher than 100 nM were too toxic for the cells. We therefore used CGP 41251, an analog of staurosporine previously shown to be less toxic [23], and studied PKC-dependent signaling pathways for gelatinase B production by macrophages. In this paper, we are reporting that CGP 41251 stimulates significant levels of gelatinase B in two different monocyte/macrophage cell lines, in a synergistic way, to the stimulation observed with LPS or PMA. Similar treatment of human blood monocytes and rat peritoneal macrophages only yielded insignificant increases of gelatinase B production. CGP 41251 alone stimulates gelatinase B production in RAW 264.7 and THP-1 cells. The CGP 41251-induced gelatinase B production is inhibited by calphostin C and by dexamethasone, and appears to be calcium-independent. On the other hand, LPS- and CGP 41251-induced gelatinase B production show a significant increase in the presence of *trans*-retinoic acid (RA), at both mRNA and protein levels. In fact, RA alone appears to be an inducer of gelatinase B production in macrophages.

## 2. Material and methods

### 2.1. Reagents

LPS (*Escherichia coli*, strain 0127:B8), gelatin (type A, from porcine skin), RA, A23187, dexamethasone and PMA were obtained from Sigma (St. Louis, MO, USA). Herbimycin, okadaic acid, genistein and all tissue culture materials were obtained from Gibco-BRL (Grand Island, NY, USA). Calphostin C, KT 5720 and KT 5823 were purchased from Kamiya Biomedical Company (Thousand Oaks, CA, USA). Ethylenebis (oxyethylenenitrio) tetraacetic acid (EGTA) was obtained from Boehringer-Mannheim (Laval, Quebec, Canada). CGP 41251 was generously donated by Ciba-Geigy (Basel, Switzerland).

### 2.2. Cell cultures and treatments

All established cell lines were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) and were grown in culture media recommended by ATCC supplemented with 5% fetal bovine serum (FBS), 10 mM HEPES buffer and antibiotics (complete medium). Cells were scraped or trypsinized, seeded at a density of $2.5 \times 10^5$ cells/ml in 24-well cluster plates, and incubated 18 h at 37°C (+5% $CO_2$). Peripheral blood monocytes were isolated from human male volunteers, as previously described [4] and peritoneal exudate cells were collected from male Fischer F344/N rats (specific pathogen-free; no inoculation) by repeated washing of the peritoneal cavity with ice-cold Hanks' balanced salt solution (HBSS). Human blood monocytes and peritoneal macrophages were adjusted to $10^6$ cells/ml in RPMI complete medium, seeded (1 ml/well) in 24-well cluster plates and further enriched by a 2-h adherence step at 37°C (+5% $CO_2$). Monolayers were washed three times with warm medium to completely remove FBS. Fresh serum-free medium containing either LPS, CGP 41251 or RA at the specified concentrations were then added to the cultures. When inhibitors were used, cells were pretreated for 30 min at 37°C (+5% $CO_2$) with varying doses of inhibitor before the addition of LPS or CGP 41251. Cells were further incubated for 18 h at 37°C (+5% $CO_2$). Supernatants were harvested and stored at −20°C until assayed. Cell monolayers were next fixed in formaldehyde (5% in PBS), washed with water and stained with crystal violet 1%.

### 2.3. Detection of gelatinase B activity

Gelatinase B activity of cell culture supernatants was determined by SDS/PAGE zymography using gelatin as a

substrate, as described by Masure et al. [9]. Briefly, aliquots of 20 μl were run on 7.5% acrylamide gels containing 0.1% gelatin for 18 h at 50 V, at room temperature. After electrophoresis, gels were washed to remove SDS, incubated 18 h at 37°C in a renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% $NaN_3$, 1% Triton X-100). Subsequently, gels were stained with Coomassie brilliant blue G-250 and de-stained in methanol/acetic acid. Gelatinase B was detected as unstained bands on a blue background. Gelatinase B activity was quantitated by computerized image analysis (BioRad, model GS-670 Densitometer, Mississauga, Ontario, Canada). Results are expressed as arbitrary scanning units.

### 2.4. Determination of nitric oxide production

NO production was determined by the evaluation of the nitrite concentration in culture supernatants, using the Griess reagent [24]. Briefly, 100 μl of culture supernatant was combined with 100 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene-diamine dihydrocloride/2.5% $H_3PO_4$) and allowed to stand at room temperature for 10 min. Absorbance was measured at 540 nm. Nitrite concentrations were determined using a standard curve obtained with $NaNO_2$.

### 2.5. Northern blot analysis

Cells were cultured in serum-free medium for 16 h prior to CGP 41251, LPS or RA stimulation, lysed with guanidium isothiocyanate and total RNA was purified by centrifugation on CsCl [25]. Aliquots of 15 μg of RNA were size fractionated in a 1.1% agarose denaturing gel containing 5% formaldehyde and transferred onto a nylon membrane (Amersham Hybond-N, Oakville, Ontario, Canada). RNA were cross-linked by U.V. and the membrane prehybridized for at least 4 h in 50% formamide, 5 × Denhardt's, 6 × SSC and 50 μg/ml of sheered denatured salmon sperm DNA. Hybridization was performed in the same solution, to which 10% dextran sulfate and $2–5 \times 10^6$ cpm/ml of denatured probe were added. Murine gelatinase B (2.0 kb) cDNA was the *Xba*I/*Asp*I fragment of the pSM2 plasmid [14]. The GAPDH (983 bp) probe was a reverse transcriptase (RT)-PCR product obtained with specific amplifiers sets (Clontech, Palo Alto, CA, USA). Probes were labelled with $^{32}$P-dCTP (ICN Biomedical, Mississauga, Ontario, Canada) using the random primer labelling kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). After 16 h, the membrane was washed twice (15 min) with 2 × SSC, 0.1% SDS at 42°C and once (15 min) with 0.2 × SSC, 0.2% SDS at 60°C, then exposed with a Kodak X-Omat Ar (Eastman Kodak Company, Rochester, NY, USA) film at −70°C with an intensifying screen. Densitometric analyses were obtained with a BioRad video densitometer (model GS-670).

## 3. Results

### 3.1. Effect of CGP 41251 on gelatinase B production by macrophages, monocytes and other cell lines

Four different types of monocytes/macrophages were treated for 24 h with a dose of 1 μM of CGP 41251, both in the presence and in the absence of LPS (Fig. 1). For the THP-1 cell line, PMA was used instead of LPS since undifferentiated THP-1 cells are unresponsive to LPS [13]. In two of the four macrophage cell types, RAW 264.7 and THP-1, culture supernatants from CGP 41251-treated cells showed a significant enhancement of the production of gelatinolytic activity when compared to untreated cells. In the case of human blood monocytes and rat peritoneal macrophages, densitometric analysis of the gels revealed insignificant increases of 10 and 25%, respectively. In all four macrophage cell types, the gelatinase band present in CGP 41251-treated samples co-migrated with that of LPS- or PMA-treated samples. In each case, the effect of CGP 41251 was additive to that of LPS or PMA. CGP 41251 was also tested with four non-macrophagic cell lines, whether in the presence or in the absence of PMA, as LPS has no effect on the gelatinase production of these cells (unpublished observations): all these cell lines produced constitutive levels of gelatinase A and three of them

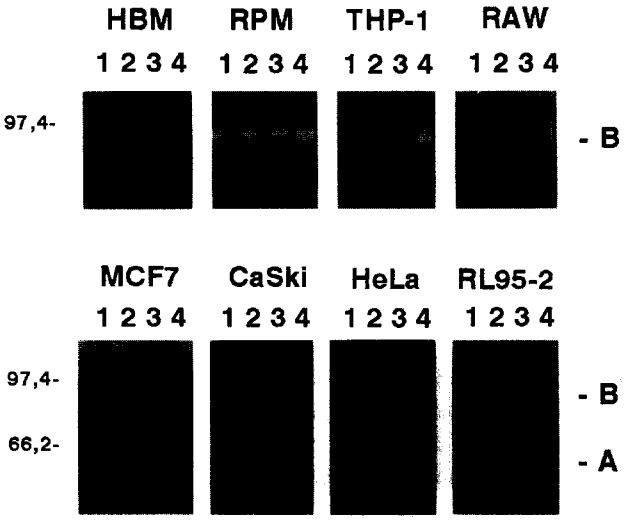


Fig. 1. Effect of LPS and CGP 41251 on the gelatinase B production of different types of monocytes/macrophages. Four different types of monocytes/macrophages (human blood monocytes (HBM), rat peritoneal macrophages (RPM), THP-1 cells and RAW 264.7 cells), and four types of nonmacrophagic tumorigenic cells (MCF7, CaSki, HeLa and RL95-2) were incubated for 24 h at 37°C (+5% $CO_2$) in serum-free conditions. Cells were left untreated (1), or were treated with a stimulator (50 ng/ml of LPS for RAW 264.7 cells, HBM and RPM or 100 nM PMA for the other cell types (2), with 1 μM CGP 41251 alone (3), or a mixed solution of LPS (or PMA) + CGP 41251 (4). Supernatants were next collected and assayed for their gelatinase A and B production (indicated right) using gelatin zymography. Molecular weight markers appear on the left in kilodaltons.

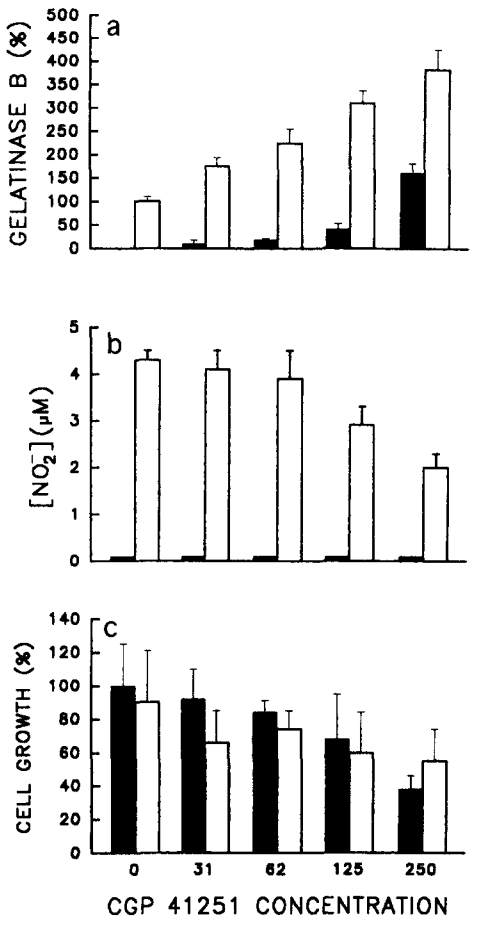


Fig. 2. Dose-response effects of CGP 41251 on gelatinase B production, nitric oxide production and growth of RAW 264.7 cells. RAW 264.7 cells were treated for 24 h at 37°C (+5% $CO_2$) with different doses of CGP 41251 in absence (filled bars) or in the presence of LPS (50 ng/ml) (empty bars). Supernatants were next collected and assayed for their gelatinase B production using gelatin zymography and densitometry (a), and for their $NO_2^-$ concentration (b). Cell monolayers were also fixed with formaldehyde 5% and stained with crystal violet for evaluation of the toxic effect of the different treatments (c). The gelatinase production of LPS-induced cells and the stain content of unstimulated cells were arbitrarily evaluated as 100%. The results are expressed as the means ± standard error (S.E.) of three different experiments.

(CaSki, HeLa and RL95-2) produced gelatinase B following PMA treatment. When tested alone, CGP 41251 had no significant effect on the production of both gelatinase A or B in any of these three cell lines (Fig. 1). In contrast to the observation with macrophages, CGP 41251 almost completely inhibited the PMA-induced production of gelatinase B in CaSki, HeLa and RL95-2 cells. Similar results were obtained with several other non-macrophagic cell lines, such as mouse B16.F1 melanoma and human Hec 1B endometrial adenocarcinoma (data not shown).

### 3.2. CGP 41251-induced gelatinase B production in RAW 264.7 cells

Mouse macrophage RAW 264.7 cells were treated for 24 h with doses of CGP 41251 ranging from 31 nM to 250 nM both in the presence or absence of an optimal dose of 50 ng/ml of LPS [22]. Analysis of the zymograms showed that CGP 41251 alone (Fig. 2a) increased gelatinase B production in a dose-dependent fashion. Moreover, the effect of CGP 41251 was synergistic with that of LPS. CGP 41251 alone had no effect on NO production of RAW 264.7 cells (Fig. 2b) and inhibited the LPS-induced NO response. When 250 nM CGP 41251 was used, a 53% decrease in the LPS-induced NO production was observed. A dose-response toxic effect was also observed when RAW 264.7 cell monolayers treated with CGP 41251 alone were next stained with crystal violet (Fig. 2c). However, no significant toxic effect was observed when RAW 264.7 cells were treated with the CGP 41251/LPS combination. The dose of 250 nM of CGP 41251 was chosen for subsequent experiments.

### 3.3. Modulation of gelatinase B production in RAW 264.7 cells induced by LPS and CGP 41251

In order to define the signal transduction pathways for gelatinase B secretion in macrophages several agonists/antagonists were tested. RAW 264.7 cells were treated for 30 min with 1 μM calphostin C, 100 nM

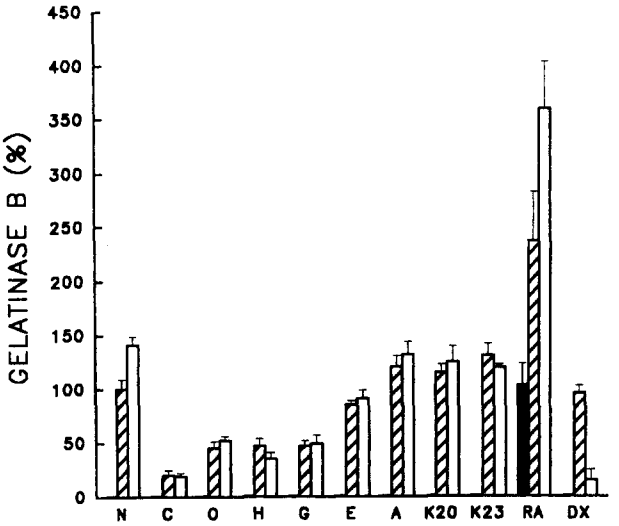


Fig. 3. Effect of different agents on gelatinase B production of RAW 264.7 cells. RAW 264.7 cells were left with no treatment (N) or treated for 30 min at 37°C (+5% $CO_2$) with 1 μM calphostin C (C), 100 nM okadaic acid (O), 1 μM herbimycin A (H), 50 μM genistein (G), 1 mM EGTA (E), 1 mM A23187 (A), 1 μM KT 5720 (K20), 1 μM KT 5823 (K23), 100 nM retinoic acid (RA) or 10 μM dexamethasone (DX) before addition of medium alone (filled bars), LPS (50 ng/ml) (hatched bars) or 250 nM CGP 41251 (empty bars). All these agents were used at optimal doses, as determined in preliminary experiments (data not shown). Supernatants were next collected and assayed for their gelatinase production using gelatin zymography and densitometry. The gelatinase B production of LPS-induced cells was arbitrarily evaluated as 100%. In the group pretreated with agent and next treated with medium alone (filled bars), detectable levels of gelatinase were observed only with RA pretreatment. The results are expressed as the means ± S.E. of three different experiments.

okadaic acid, 1 μM herbimycin A, 50 μM genistein, 1 mM EGTA, 1 mM A23187, 1 μM KT 5720, 1 μM KT 5823, 1 μM RA, or 10 μM dexamethasone before the addition of LPS or CGP 41251. Only RA induced gelatinase B production when tested alone (Fig. 3). Moreover, the effect of RA was additive to that of CGP 41251 and LPS. Calphostin C inhibited gelatinase B production induced by LPS and CGP 41251 by more than 80%. Okadaic acid, herbimycin A and genistein partially inhibited these responses (approx. 50%). EGTA, A23187, KT 5720 and KT 5823 had no significant effect. For EGTA pretreatment, the culture medium was changed 6 h after LPS addition in order to avoid the inhibitory effect of EGTA on gelatinase activity. Dexamethasone inhibited the CGP 41251-induced gelatinase B production by 80% without affecting the LPS-induced response in a significant manner. None of the agents used had a significant toxic effect, except for calphostin C and genistein, which gave an 80% and a 50% decreased response, respectively.

### 3.4. Dose-response effect of retinoic acid on gelatinase B production in RAW 264.7 cells

RAW 264.7 cells were treated for 20 h with doses of RA ranging from $10^{-12}$ to $10^{-5}$ M, either in the presence or absence of LPS or CGP 41251. RA alone (Fig. 4) stimulated gelatinase B production of RAW 264.7 cells starting at $10^{-10}$ M and reaching a plateau at $10^{-7}$ M.

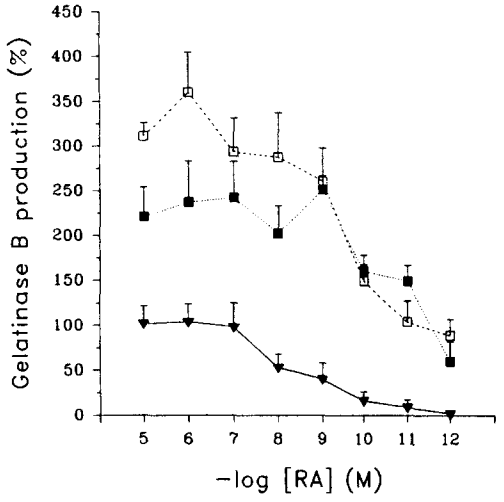


Fig. 4. Dose-response effect of *trans*-retinoic acid on gelatinase B production of LPS- and CGP 41251-induced RAW 264.7 cells. RAW 264.7 cells were treated for 30 min at 37°C (+5% $CO_2$) with different doses of RA before addition of medium alone (filled triangles), LPS (50 ng/ml) (empty squares) or 250 nM CGP 41251 (filled squares). Supernatants were next collected and assayed for their gelatinase production, using gelatin zymography and densitometry. The gelatinase production of LPS-induced cells was arbitrarily evaluated as 100%. The gelatinase production of CGP 41251-induced cells represented 130% of the LPS-induced response (results not shown in the figure). The curves represent the means ± S.E. of three different experiments.

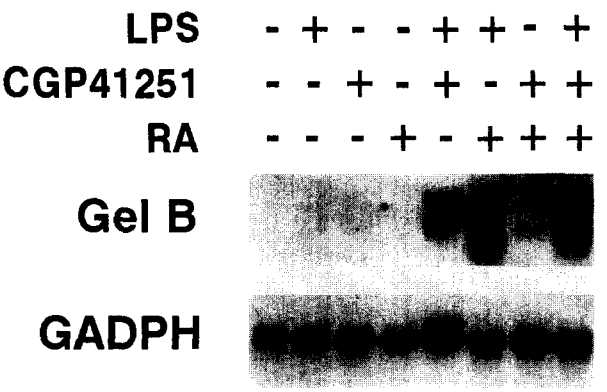


Fig. 5. Effect of LPS, CGP 41251 and *trans*-retinoic acid on gelatinase B mRNA levels in RAW 264.7 cells. RAW 264.7 cells were treated for 18 h at 37°C (+5% $CO_2$) with LPS (50 ng/ml), 250 nM CGP 41251 and/or 100 nM RA. Total RNA was extracted and aliquots of 15 μg were hybridized with $^{32}$P-labelled probes specific for mouse gelatinase B and GADPH.

This effect of RA was synergistic with that of LPS and CGP 41251, starting from $10^{-11}$ M of RA.

### 3.5. Effect of LPS, CGP 41251 and retinoic acid on RAW 264.7 gelatinase B mRNA levels

We have previously demonstrated [22] that an 18-h treatment with LPS was optimal in increasing the steady-state levels of gelatinase B mRNA in RAW 264.7 cells. Therefore, these cells were treated for 18 h with optimal doses of LPS, CGP 41251 and RA, either alone or in combination. Total RNA was extracted from the cells and hybridized with a probe specific for mouse gelatinase B (Fig. 5). Densitometric analysis of the blots indicated a barely detectable increase of gelatinase B mRNA level in the CGP 41251-treated cells (15% of the LPS response) and no effect on RA-treated cells. However, the combinations CGP 41251 with LPS, RA with LPS and CGP 41251 with RA gave 10-, 6- and 14-fold higher responses than LPS alone, respectively. Moreover, a combination of the three agents gave a 17-fold higher response, when compared to LPS alone. Zymographic analysis of the culture supernatants of the same specimens confirmed that CGP 41251 and RA had comparable effects to that of LPS at the protein level (data not shown).

## 4. Discussion

Macrophages use matrix remodelling proteinases in the process of migration. In addition, these enzymes could possibly be at the biochemical basis of autoimmunity. The present study was undertaken to define regulatory pathways in the modulation of gelatinase B activity by macrophages.

Similar to many other cell types, macrophages can secrete two types of gelatinases: gelatinase A (±70 kDa,

MMP-2) and gelatinase B (±90 kDa, MMP-9). Both enzymes are differently regulated in human and mouse primary macrophages and macrophagic cell lines. In this study, we focussed on the regulation of gelatinase B activity. All our experiments were done in serum-free medium to eliminate the gelatinolytic activity [5] and the possible modulatory factor(s) [26] contained in sera. A significant increase of gelatinase B production and a superinduction of this response induced by LPS and PMA have been observed in two different types of murine and human macrophages (RAW 264.7 and THP-1) treated with CGP 41251, a member of the staurosporine family of PKC inhibitors. This effect of CGP 41251 was not significant in human blood monocytes and rat peritoneal macrophages, although some induction was observed by densitometric analysis of the gels. Considering that human monocytes and rat peritoneal macrophages represent enriched, but not pure, preparations of cells, these ambiguous results are not surprising. For instance, the presence of contaminating cells in these preparations, or the use of macrophages at different stages of differentiation, may explain this relative absence of stimulation when compared to RAW 264.7 and THP-1 cells. However, in no case did CGP 41251 inhibit gelatinase production of the four macrophage cell types studied, as it did for PMA-induced non-macrophagic tumor cell lines. The inhibitory effect of CGP 41251 on tumorigenic cell lines was expected since it has been previously described by our group [27] and others [28], using staurosporine.

Therefore, considering that: (1) RAW 264.7 cells are inducible by LPS, the most studied macrophage stimulator; and (2) RAW 264.7 cells can accomplish many recognized macrophage functions [18–22], our results suggest that the RAW 264.7 cell line is representative of the macrophage, or at least of some subsets of this cell type, regarding the effect of CGP 41251 on the gelatinase B production. In RAW 264.7 cells, CGP 41251 acted in synergy with LPS for gelatinase B production, at both the mRNA and protein level, but concomitantly inhibited the LPS-induced NO production. Presently, we cannot rule out the possibility that the stimulation of gelatinase B production and the inhibition of NO production may be related to the toxicity of CGP 41251. However, the link between gelatinase B and toxicity of CGP 41251 appears improbable since staurosporine, which is reportedly more toxic than CGP 41251 [23], showed no stimulatory effect on the gelatinase B production in RAW 264.7 [22]. Gelatinase B production induced by LPS and CGP 41251 was also superinduced by RA. In fact, RA alone, which had no toxic effect on RAW 264.7 cells (data not shown), appeared to be a gelatinase B inducer. Moreover, the triple combination of LPS with CGP 41251 and RA showed a higher response than any of these agents tested either alone or in double combination. Northern blots were performed at the optimal time of gelatinase B mRNA induction by LPS [22]. In the presence of LPS, two mRNA transcripts were detected by the mouse cDNA probe, confirming the results obtained by our group [14,22] and by others [29]. Our results (Fig. 5) revealed a barely detectable effect of CGP 41251, and no visible effect of RA on the steady-state levels of gelatinase B mRNA. A number of factors involved in the regulation of PMA- and LPS-induced responses, such as Fos and I$k$B, could play a role in this regulation since these responses are known to possess negative regulatory activities which can be controlled through their phosphorylation state [30,31]. Regarding the potentiating effects of LPS and RA, members of NF-$k$B (reviewed in Ref. [32]) and RA receptor (RAR) families (reviewed in Ref. [33]) represent good candidates for mediating the observed effects. Analysis of the known promoter regions of human [34] and murine [14] gelatinase B genes showed the presence of a NF-$k$B-binding element (GGAATTCCCC) in the 5′ promoter region of both genes. No RA responsive element (RARE; AGGTCAnnnAGGTCA) has been found in any of these two genes; however, as shown by Kitabayashi et al. [35], RA can bind to other sequences. In the case of the murine gelatinase B gene [14] the 5′ promoter region contains five AGGTCT sites which differ by only one nucleotide from a consensus AGGTCA half-site potentially recognized by RAR and the thyroid receptor. RA may also act indirectly, through inhibition of a negative regulatory transcription factor. For instance, human RAR$\alpha$ has been shown to possess a 'silencing' domain in its C-terminal part; in the absence of RA, this domain can inhibit gene transcription, a process which can be cell type-specifically neutralized by RA [36]. Several other mechanisms of negative regulation of transcription, such as competition between nuclear factors or cross-talk between receptors and protooncoproteins are also possible (reviewed in Ref. [37]), particularly if the presence of two AP1 sites in both the human and murine gelatinase B promoter regions is considered. Finally, we cannot exclude a dual action of CGP 41251, RA and/or LPS on both positive and negative mechanisms of regulation [38].

The pretreatment of RAW 264.7 cells with different agents revealed that gelatinase B production induced by LPS and CGP 41251 shared common characteristics: (a) it was inhibited by calphostin C, one of the most selective PKC inhibitors (reviewed in Ref. [39]). Accordingly, this response appears to be PKC-dependent. Therefore, it seems that CGP 41251 would rather act as a PKC agonist, an observation which has been made by others, especially in the polymorphonuclear cell model [40–42]. CGP 41251 may fail to inhibit the calcium-independent PKC isoforms while inhibiting the calcium-dependent isoforms [43,44]. If the ratio of calcium-dependent/calcium-independent PKC isoforms in macrophage is different from that of non-macrophagic tumor cells, the overall effect of CGP 41251 on gelatinase B production would be the opposite. On the other hand, CGP 41251 may also act on another enzyme, such as phospholipase D [42]. Finally, CGP 41251 may inhibit only the phosphorylative activities of PKC without

affecting its other potential regulatory activity, as proposed for the activation of phospholipase D by staurosporine [45]; (b) gelatinase B production was partially inhibited by okadaic acid, a phosphatase inhibitor; (c) it was also partially inhibited by genistein and herbimycin A, two tyrosine-specific protein kinase inhibitors; (d) gelatinase B production was not inhibited in the presence of KT 5720 and KT 5823, two inhibitors of cAMP- and cGMP-dependent protein kinases; (e) finally, even though intracellular calcium had been modulated in a negative or a positive way (using EGTA and A23187, respectively), there was no significant change on gelatinase B production, indicating a calcium-independent mechanism. Furthermore, using dexamethasone, a major difference was demonstrated between LPS and CGP 41251: LPS-induced gelatinase B production was not affected, but CGP 41251-induced response was inhibited by more than 80%, suggesting that glucocorticoid receptors are involved in the down-regulation of CGP 41251-induced gelatinase B production. Transformation of RAW 264.7 cells with different plasmids containing one of these regulatory factor cDNAs under the control of a strong promoter, would certainly yield additional interesting results.

In many cell types, including macrophages, MMP genes such as those coding for gelatinase B, collagenase and stromelysin, but not gelatinase A, respond in a similar fashion to LPS and PMA stimulation [12,16,27,46]. Furthermore, these genes contain *cis*-acting elements, such as AP1- and NF-kB-binding sites, in their promoter region (reviewed in Ref. [6]). Until now, relatively few result have been reported with other MMPs regarding PKC inhibitors. Staurosporine was reported to inhibit stromelysin production induced by nerve growth factor in PC12, a rat pheochromocytoma cell line [47]. However, a number of studies were performed on the inhibitory effect of RA and dexamethasone on different constructs containing the promoter region of collagenase and the CAT reporter gene [35,48]. Regarding gelatinase B, staurosporine was shown to induce it in human neutrophils [40]. Recently, it was reported that staurosporine stimulated the expression of gelatinase B and collagenase in rat mucosal keratinocytes and that dexamethasone inhibited gelatinase B production induced by transforming growth factor $\alpha$ and epidermal growth factor, but not by PMA [49].

Our results raise interesting questions regarding the therapeutic use of PKC inhibitors and RA in cancer therapy and prevention trials, as well as in inflammatory diseases, particularly since a number of reports suggest that gelatinase B might play a role in metastasis development (reviewed in Refs. [6,7]) and in inflammatory processes [8]. In all nonmacrophagic tumor cell lines that we and others [27,50] have tested so far, gelatinase B production was always inhibited by PKC inhibitors such as calphostin C, staurosporine and CGP 41251. Because gelatinase B production of tumor-infiltrating macrophages would simultaneously be increased by these treatments, it would remain possible for 'metastasizing' tumor cells to use this enzyme to break the surrounding ECM [7,51]. Our results also suggest that agents such as CGP 41251 and RA can inhibit LPS-inducible products possessing tumoricidal and inflammatory properties, such as TNF-$\alpha$ and NO, while concomitantly stimulating the production of gelatinase B and, possibly, other molecules implicated in inflammatory diseases and cancer. Therefore, the search for substances that would inhibit or carefully balance the production of all these factors could potentially improve the benefits of such types of treatment.

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