Original Research Article (Experimental)

Involvement of tyrosine-specific protein kinase and protein kinase C in J774A.1 macrophage functions activated by *Tinospora cordifolia*

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A B S T R A C T

Background: Macrophages are the first line of defense and constitute important participant in the bi-directional interaction between innate and specific immunity. Macrophages are in a quiescent form and get activated when given a stimulus. In our previous studies we have reported that guduchi or LPS treatment of macrophages enhanced production of nitric oxide (NO) and increased tumoricidal activity against L929 fibroblast cells.
Objective: In the present study effect of *Tinospora cordifolia* commonly known as guduchi on macrophage activation and the mechanism of action i.e. involvement of protein kinase C inhibitor and tyrosine-specific protein kinase inhibitor was investigated.
Materials and Methods: The present study was undertaken to determine whether H-7 (inhibitor of protein kinase C) and/or genistein (inhibitor of tyrosine-specific protein kinase) could inhibit guduchi or LPS-induced macrophage NO and TNF-α production or reduce the cytolysis of L929 fibroblast cells.
Results: It was observed that *in vitro* incubation with H-7 and/or genistein completely inhibited guduchi or LPS-induced NO and TNF-α production by macrophages (J774A.1).
Conclusion: The inhibitory effects of H-7 and/or genistein, suggest that phosphorylation via these kinases may upregulate the NO synthase activity in macrophages.
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1. Introduction

Macrophages are quiescent cells, which get activated when stimulated. Different types of agents such as antibiotics, anti-metabolites and cytokines may exert an immunomodulating action that is expressed in the augmentation and/or inhibition of different immune responses [15]. One of the most promising recent alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defense responses [23]. Number of natural products and synthetic immunopotentiators termed as Biological Response Modifiers (BRMs) are becoming increasingly popular for their potential in augmenting immune responses. Among the natural BRMs many herbs and medicinal plants have long been known for their immunoaugmentary potential; however, only recently scientists have recognized them for their possible BRM actions. The herb guduchi isolated from botanical sources have attracted a great deal of attention in the biomedical arena because of its broad spectrum of therapeutic properties and relatively low toxicity. Our study is based on the investigation of the BRM *Tinospora cordifolia* (guduchi) as an activating agent of macrophages *in vitro* and the bacterial endotoxin LPS as a positive control.

In the present study two different inhibitors (H-7), an inhibitor of protein kinase C and/or (genistein), a tyrosine-specific protein kinase inhibitor were used to analyze the regulation of guduchi or LPS mediated macrophage activation. Protein kinase C (PKC) has been shown to be a signal transducer during tumorigenesis, tumor cell invasion, and metastasis. Recent studies have reported that the PKC inhibitor, 7-hydroxystaurosporine (H-7), inhibits tumor cell invasion [22]. Genistein, an isoflavone, has been shown to inhibit cell proliferation and enhance apoptosis in cancer cells. Accumulating bodies of evidence suggest that genistein is expected to synergistically promote the anti-proliferative effects of chemotherapeutic agents on neoplasia without toxicity [24]. In the present study involvement of these specific kinases, in

guduchi or LPS-mediated macrophage activation has been investigated.

Macrophage activation is known to occur through a series of stages ranging from a level equivalent to resident tissue macrophages and culminating at an activated state whereupon macrophages become competent to kill several pathogens and lyse tumor cells [1,14]. Although activated macrophages produce a number of physiologically active molecules with cytotoxic and/or cytostatic effects, only interleukin-1 (IL-1), tumor necrosis factor (TNF) and reactive nitrogen intermediates (RNI) have been clearly implicated in monocyte/macrophage mediated tumor cytotoxicity [2,5,8,9,13,21].

Nitric oxide (NO) is formed enzymatically from a terminal guanidine-nitrogen of L-arginine by the so called NO synthases (NOSs) that yield L-citrulline as a co-product [16,17]. Both in the NO producing cell and in specific NO target cells, NO functions as the first messenger of nitrinergic signal transduction, activating GC-S [GTP pyrophosphate-lyase (cyclizing)] [3] and thereby increasing the intracellular concentration of the second messenger molecule cGMP [4,18]. Whether the complete pathway operates in macrophages has not been investigated. In guduchi activated macrophages NO biosynthesis could also be the mediator of macrophage-mediated tumor cytotoxicity [7,9–11,21]. While our understanding of the mechanism of action of this BRM is still developing, it appears that the primary mechanism involves induction of the immune system. Our previous studies demonstrate that the basic mechanism of the immunostimulatory, antitumor, bactericidal and other therapeutic effects of guduchi is thought to occur via macrophage stimulation [10–12]. We have focused this study on the involvement of protein kinase C and tyrosine-specific protein kinase in the BRM (guduchi) or LPS-mediated macrophage functions. The purpose of the present study was to investigate whether H-7 (inhibitor of protein kinase C) and/or genistein (inhibitor of tyrosine-specific protein kinase) could decrease macrophage derived NO and TNF-α production in the setting of an *in vitro* herbal (guduchi) treatment or endotoxin (LPS) challenge.

2. Materials and methods

Reagents: Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and 25 mM HEPES buffer were purchased from (HiMedia Pvt. Ltd. India.) Fetal bovine serum was purchased from Hyclone (Logan, USA) and heat inactivated at 56 °C for 30 min. The whole plant extract of *T. cordifolia* was used for the study. The plant was obtained from medicinal plant nursery, Pune, Maharashtra. The plant was subjected to extraction with 200 ml methanol at 50 °C for 8 cycles by Soxhlet extraction process. The extract was then concentrated with rotator vacuum evaporator and used for further analysis. For enzymatic analysis, fresh crude extract with phosphate buffer (pH 7) was used. The guduchi extract prepared in incomplete DMEM were tested for endotoxin contamination by limulus amebocyte lysate assay which showed insignificant levels [0.0007 ng/mg]. Necessary precautions were taken to avoid endotoxin contamination through out the investigation, by using endotoxin free buffers, reagents and sterile water. All other chemicals and solvents used in this study were obtained from Sigma Chemical Company (St. Louis, USA) and were of analytical grade or the highest grade available.

Cells: The macrophage J774A.1 and the fibroblast L929 cell lines were obtained from National Center for Cell Sciences (NCCS, Pune). J774A.1 cell line was used as source of macrophages, (Origin: BALB/c mouse; Nature: Mature) grown and maintained in the Dulbecco's Modified Eagle Medium (DMEM) (pH 7.5) enriched with 10% fetal bovine serum, at 37 °C and 5% CO_2.

2.1. Viability assay

Cell viability was determined by the trypan blue dye exclusion technique. Equal volumes of cell suspensions were mixed with 0.4% trypan blue in PBS, and the unstained viable cells were determined. These cells were further used for cytotoxicity assay in 2×10^6 densities per ml in the 96 well tissue culture plates.

Stimulation of macrophages: The macrophage cells (cell line J774A.1) from late log phase of growth (subconfluent) were seeded in 96 well flat bottom microtiter plates (Tarsons, India) in a volume of 100 μl under adequate culture conditions. Guduchi (80 μg/well) or LPS (10 μg/well) were added in a volume of 100 μl with and/or without inhibitors in triplicate. The cultures were incubated at 37 °C and 5% CO_2 environment. After 24 h and 48 h incubation percent viability was checked and culture supernatants were collected and assayed for nitric oxide and TNF-α activity.

2.2. Inhibitor treatment to the macrophages as second messengers

Along with guduchi or LPS macrophage cells were treated with two different inhibitors, H-7 (inhibitor of protein kinase C) and/or genistein (inhibitor of tyrosine-specific protein kinase), within a concentration of 10 μM/ml, respectively. Supernatant collected from these cells were assayed for nitrite generation and for tumoricidal activity to check inhibition.

2.3. Nitrite assay

The concentration of stable nitrite, an end product of the nitric oxide present in the supernatant of treated or untreated J774A macrophage cell cultures (2×10^6 cells/ml), was measured by the method of Ding et al. [6] based on the Griess reaction [20]. Briefly, 50 μl of supernatant was incubated with an equal volume of Griess reagent (1% sulphanilamide in 2.5% H_3PO_4 and 0.1% naphthyl-ethylene-diaminedihydrochloride in distilled water; both solutions mixed in a ratio of 1:1 at room temperature) for 10 min. The absorbance at 550 nm was then measured in a microtitre plate reader. The standard curve for nitrite was prepared by using 10–100 μM sodium nitrite in distilled water.
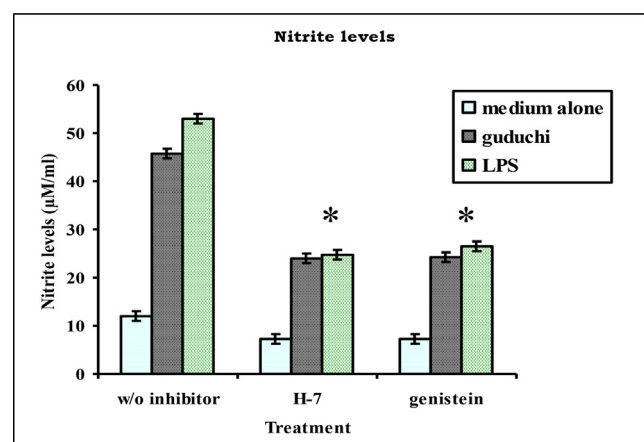

Fig. 1. Nitrite levels in macrophages (J774A.1) after treatment with guduchi or LPS along with the inhibitors. Values represent nitrite content in μM. Bars shows mean ± SD for triplicate cultures. Data are representative of three separate experiments (*$p < 0.05$; versus value of guduchi or LPS treated macrophages) [w/o: without inhibitor].
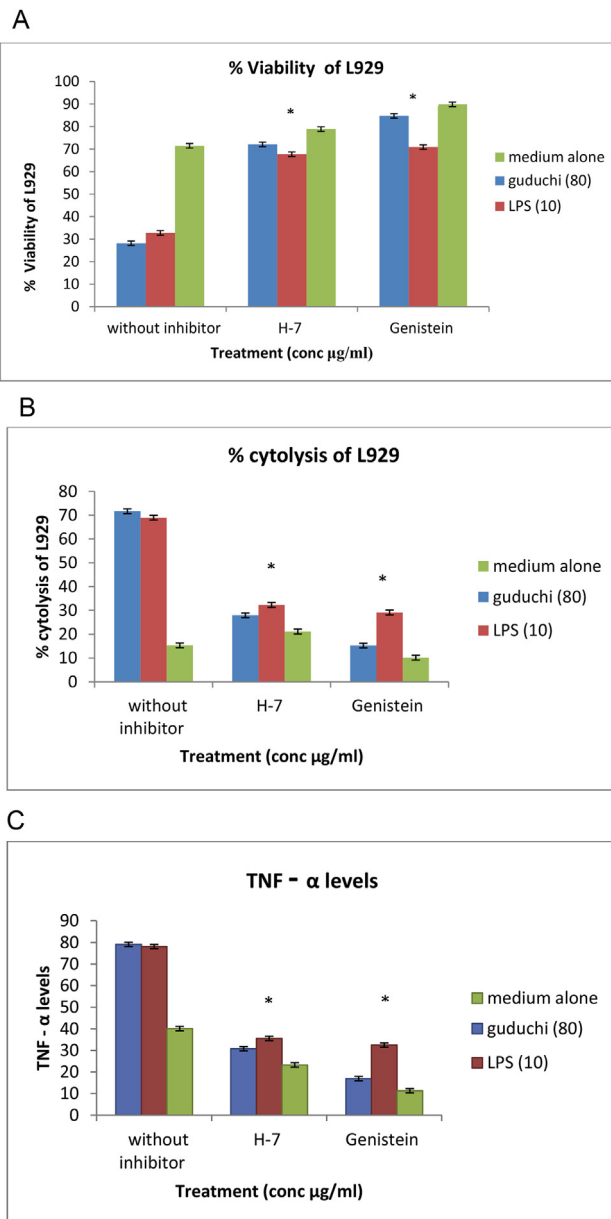
A

B

C

Fig. 2. Involvement of second messengers' protein kinase C and tyrosine-specific protein kinase in macrophage activation using specific inhibitors (H-7 and Genistein).

2.4. Assay for TNF activity

The activity of TNF-α in the culture supernatants of guduchi or LPS and inhibitor (H-7 or genistein 10 μg/ml each) treated and untreated macrophages was measured by a modification of the Mosmann method based on the reduction of MTT (Sigma) to a colored formazan by living cells [18]. Briefly, 2×10^6 L929 cells, in 100 μl complete medium were grown in wells of a 96 well tissue culture plate in the presence of 1 μg/ml of actinomycin D and 100 μl of test culture supernatant. Cell viability was assessed after 24 h of incubation. The supernatant was discarded and 10 μl of MTT; 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (3 mg/ml) was added to each well and plates were further incubated for 2 h at 37 °C. The enzyme reaction was then stopped by addition of 150 μl of dimethyl sulfoxide (DMSO). Plates were incubated for 10 min under agitation at room temperature and colorimetric measurement of the formazan was performed on an enzyme-linked

immunosorbent assay plate reader at 570 nm. Cells treated with culture supernatants of untreated macrophages were consider as control. Percent viability and percent cytolysis of these cells was then calculated by the given formula.

$$\text{Percent viability} = \frac{E}{C} \times 100$$

where, E is the absorbance of cells treated with culture supernatants of guduchi or LPS treated and untreated macrophages and C is the absorbance of cells treated with medium alone.

$$\text{Percent cytolysis} = (1 - \text{Percent viability})$$

Three independent experiments in triplicate were performed for the determination of TNF-α in the supernatants. TNF-α (mouse origin) in concentration of 10–100 pg was used as standard. The TNF-α levels were then calculated from the standard curve.

2.5. Statistical analysis

Statistical significance of difference between the control and experimental samples were calculated by Student's t-test and one way ANOVA. All the experiments were done in triplicate samples. Conclusion was drawn from 3 independent experiments.

3. Results

3.1. Involvement of second messengers in macrophage activation

In our previous study we had reported activation of macrophages by guduchi or LPS treatment. Elevated TNF-α level was found in guduchi or LPS treated macrophage cell supernatants after 24 h as compared to macrophage treated with medium alone [10–12]. A significant decrease ($p < 0.05$) in nitrite levels by macrophages treated with guduchi or LPS along with H-7 (inhibitor of protein kinase C) and/or genistein (inhibitor of tyrosine-specific protein kinase) was observed (Fig. 1). Also, inhibitor treatment led to increased tumor viability and decreased tumor cell cytolysis *in vitro* as compared to the previous results (Fig. 2A and B). Activation of macrophages is found to be reduced significantly after inhibitor treatment.

3.2. Nitrite levels

To determine whether genistein and/or H-7 inhibit the guduchi or LPS-induced NO production, nitrite assay was performed with macrophages (J774A.1) as mentioned in the materials and methods. Fig. 1 represents the NO levels in the supernatants, from macrophages pre-treated *in vitro* with guduchi or LPS and subsequently exposed to H-7 and/or genistein (Fig. 1).

Macrophages treated with BRM (guduchi) or endotoxin (LPS) showed significantly enhanced nitrite levels (45.65 μM/ml or 52.91 μM/ml, respectively) as compared to macrophages treated with medium alone (12 μM/ml). When macrophages were treated with guduchi or LPS along with the inhibitors H-7 and/or genistein, some elevation in nitrite level was observed. However, overall significant low nitrite levels in the supernatants of macrophages treated with inhibitors was observed. Guduchi when incubated with macrophages along with the inhibitors H-7 and/or genistein (23.91 μM/ml and/or 24.34 μM/ml) of nitrite levels were found, respectively. Whereas, LPS treatment to macrophages along with the inhibitors (H-7 and/or genistein) showed (24.78 μM/ml and/or 26.52 μM/ml) nitrite levels, respectively.

Also reduction in the generation of nitrite was found in macrophages treated with medium alone and inhibitors (H-7 or genistein) which were (7.21 μM/ml and/or 7.15 μM/ml) (Fig. 1).

3.3. TNF-α activity

As per our previous results [9], guduchi or LPS treated macrophage cell supernatants showed enhanced TNF-α levels and eventually enhanced cytolysis of L929 fibroblast cells as compared to medium alone. The guduchi or LPS treated macrophage cell supernatants when incubated with the fibroblast cell line (L929) for 24 h showed 70—75% or 65—70% cytolysis, respectively. However, only 20—25% cytolysis was observed in cells treated with medium alone [9]. In the present study a major difference in these parameters of macrophage activation was observed after inhibitor treatment. Macrophages when treated with guduchi along with the inhibitor (H-7) and/or genistein, for 24 h only 27.91% and/or 15.26% cytolysis was seen, respectively. LPS and H-7 treated macrophages showed (32.28%) cytolysis and LPS and genistein treated macrophages showed (29.10%) cytolysis which was found to be reduced significantly (Fig. 2B). TNF-α levels were calculated from the cytotoxicity assay as mentioned in the materials and methods. TNF-α levels after guduchi, LPS or medium alone treatment were reported previously as 80.46, 71.26 or 24.70 pg/ml, respectively [10]. When macrophages treated with guduchi along with the inhibitors H-7 or genistein, generation of TNF-α was significantly reduced 30.75 pg/ml and/or 16.95 pg/ml, respectively. When macrophages treated with LPS along with the inhibitors H-7 or genistein, showed 35.56 pg/ml and/or 32.43 pg/ml TNF-α levels, respectively (Fig. 2C).

4. Discussion

Macrophages activated with various stimuli, like bacterial endotoxin, lymphokines and BCG infection, elaborate production of nitrites/nitrates [19,20] and further, nitric oxide generated during the conversion of arginine to nitrites/nitrates, is involved in the macrophage- mediated cytotoxicity [9,21]. The present investigation was carried out to gain insight into several issues pertinent to production and regulation of NO by murine macrophages *in vitro* by guduchi or LPS treatment. In the previous study we had reported guduchi-induced production of NO, TNF-α and tumoricidal activity of macrophages (J774A.1) against the fibroblast cell line (L929) [10]. The level of nitrite measured in our assay was indicative of nitric oxide production, which is supposed to be one of the key molecules involved in the tumoricidal activity. Our results suggest more than one mechanism of tumor cell killing by macrophages activated with guduchi or LPS, as the tumoricidal activity against L929 cells is independent of nitric oxide pathway. The results were further strengthened by the observations that inhibition of NO pathway had a different effect on the culture supernatant-mediated tumor cell lysis. Earlier, we had reported that TNF-α plays a crucial role in macrophage mediated tumor cell killing [10]. Our results demonstrated that activated macrophages express the cytolytic mechanism mediated by NO and TNF-α. In the present study we have checked the role of specific inhibitors, genistein (a tyrosine-specific protein kinase inhibitor) and/or H-7 (a protein kinase C inhibitor) in tumoricidal functions of activated macrophages. Genistein (a tyrosine specific protein kinase inhibitor) and/or H-7 (a protein kinase C inhibitor) significantly inhibited guduchi/LPS induced NO release, suggesting that phosphorylation via these kinases may upregulate NOS activity. Inhibition in the production of NO, TNF-α and cytolysis mediated by macrophages was observed after treatment with these inhibitors. This indicates that tyrosine-specific protein kinase and protein kinase C have major role to play in

tumoricidal function. Inhibition of these kinases eventually inhibits macrophage activation.

5. Conclusion

Treatment of macrophages with protein kinase C inhibitor (H-7) and tyrosine-specific protein kinase inhibitor (genistein) along with the incubation of BRM, inhibited the BRM induced-tumoricidal activity of macrophages as well as production of TNF-α and NO. From this study it can be concluded that protein kinase C and tyrosine-specific protein kinase plays an important role in tumoricidal function mediated by guduchi or LPS activated macrophages.

Conflict of interest

None declared.

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