Scavenger receptor-mediated delivery of muramyl dipeptide activates antitumor efficacy of macrophages by enhanced secretion of tumor-suppressive cytokines

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Abstract: We showed that muramyl dipeptide (MDP) conjugated to maleylated bovine serum albumin (MBSA) was internalized by macrophages (Mϕ) through scavenger receptor (SCR)-mediated endocytosis, which leads to 50-fold higher cytotoxic activity against non-Mϕ tumor cells compared with that elicited by free MDP-treated Mϕ. The enhanced cytotoxic effect of MBSA-MDP was found to be a result of higher secretion of interleukin (IL)-1, IL-6, tumor necrosis factor α (TNF-α), and nitric oxide (NO) because the addition of antibodies directed against IL-1, IL-6, or TNF-α in combination with Mϕ cultures totally abrogated the tumoricidal activity of MBSA-MDP. It is interesting to note that MBSA-MDP triggers the secretion of IL-12, whereas IL-10, a Mϕ suppressor cytokine, could be detected only on free MDP treatment. The cytotoxic activity of MBSA-MDP was inhibited by indomethacin, indicating a regulatory role for prostaglandin E_2 (PGE_2). Efficient SCR-mediated intracellular delivery of MDP leading to elimination of cancer cells suggests the immunotherapeutic potential of this approach for treatment of neoplasia. *J. Leukoc. Biol.* 67: 683–690; 2000.

Key Words: *receptor-mediated endocytosis · macrophage activation · antitumor properties*

INTRODUCTION

Activated macrophages play an important role in host defense against primary and metastatic cancers. Modulation of macrophage (Mϕ) metabolism to an activated tumoricidal state is therefore an attractive alternative to conventional chemotherapy [1]. Immunomodulatory compounds such as interferons, interleukins, colony-stimulating factors, and bacterial products like muramyl dipeptide (MDP) and trehalose dimycolate have been reported to augment the tumoricidal activity of macrophages [2–5]. Recombinant cytokines in combination with tumor-specific antigens have been used to boost the antitumor immune response based mainly on T lymphocyte activation [4, 5]. Among the biological response modifiers, MDP is the smallest biomolecule possessing immunoregulatory activity [3, 6, 7]. Though a powerful inducer of the antitumor activity of macrophages, MDP is quickly eliminated from the body, thereby limiting its therapeutic utility [8]. Liposomes or soluble carrier systems based on mannosylated neoglycoproteins, acetylated low-density lipoprotein, gelatin, as well as antibodies against Mϕ surface antigens have been used to enhance the therapeutic efficacy of MDP with some degree of success [9–13]. However, the mechanism of activation of tumoricidal properties of Mϕ in such targeting regimens is not clear.

We have previously demonstrated that scavenger receptor (SCR)-mediated intracellular delivery of conjugates of the anticancer agent daunomycin with maleylated bovine serum albumin (MBSA) elicits selective toxicity toward neoplastic cells of Mϕ lineage, whereas the receptor-negative cells remained unaffected both *in vitro* and *in vivo* [14–16]. To generalize the use of the receptor system in combating neoplasms of diverse origin, intracellular delivery of biological response modifiers was envisaged. We report in this investigation that SCR-mediated intracellular delivery of MDP activates Mϕ anti-tumor activity by triggering the secretion of cytokines and other soluble mediators leading to elimination of cancer cells.

MATERIALS AND METHODS

Materials

N-acetyl muramyl L-alanyl D-isoglutamine was procured from Fluka Chemika Bio-Chemika (Switzerland). Bovine serum albumin (BSA), monensin, fucoidin, polyinosinic acid, polyguanilic acid, polycytidilic acid, fetuin, maleic anhydride, gentamicin, and fluorescein isothiocyanate (FITC)-labeled antibodies were purchased from Sigma Chemical (St. Louis, MO). Enzyme immunoassay (EIA) kits for the detection of interleukins were procured from Genzyme Diagnostics (Cambridge, MA). The EIA system for detection of prostaglandin E_2 (PGE_2), [^{3}H]dThd, and $Na^{125}I$ were purchased from Amersham International (Amersham, UK). Pyrochek-Cal, endotoxin detection kit containing amebocyte lysate, was purchased from Manukirti Biogems (Bangalore, India). Other reagents used were of analytical grade. The animals used in this study were obtained from the Small Animal Facility of the Institute and were in good health without any apparent infection as routinely tested.

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Cells

J774A.1, L929, and B16F10 cell lines were obtained from American Type Culture Collection and maintained in RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum and gentamicin (50 μg/mL). Peritoneal resident cells were harvested from 8-week-old normal healthy C57BL/6 mice by intraperitoneal injection of 8–10 mL of 10 mM phosphate-buffered saline (PBS), pH 7.2, and draining the peritoneal fluid by gravity with an 18-gauge needle. Subsequently, cells were washed twice and seeded on plastic Petri dishes in RPMI 1640 medium supplemented with 10% fetal calf serum and gentamicin for 2 h at 37°C. Nonadherent cells were removed by washing with PBS. Adherent cells were also removed from the plates by treatment with chilled 1 mM EDTA, counted, and plated in six-well plates at a density of 1×10^6 cells per well or in 96-well plates at a density of 2×10^5 cells per well. Cells were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air.

Preparation of MBSA-MDP conjugates

MDP was conjugated to BSA by using water-soluble carbodiimide in the presence of *N*-hydroxysulfosuccinimide (sulfo-NHS). Briefly, 10 mg of the MDP was incubated in the presence of 3.6 mg of EDC and 3.2 mg of sulfo-NHS in 0.1 mL of water for 10 min at room temperature to generate an active ester of MDP. Subsequently, activated MDP was reacted with 10 mg of BSA in 0.2 M sodium carbonate buffer, pH 9.5, for 1 h at room temperature. BSA-MDP conjugate was purified through Sephadex G25 column chromatography, and purified conjugate was maleylated with maleic anhydride at a pH of 8.0 as described [17]. MBSA-MDP conjugate was separated from the residual small molecules by extensive dialysis against 10 mM PBS, pH 7.3, before further characterization. MDP was estimated by a modification of the method of Levvy and McAllen for the determination of hexosamines [18]. Extent of maleylation was quantified using trinitrobenzenesulfonic acid (TNBS) as described by Habeeb [19]. A conjugate with a 15:1 molar ratio of MDP to protein was used in these studies based on the affinity of the conjugate toward the receptor system as well as the amount of MDP available for bio-efficacy. Endotoxin contamination in the conjugate was detected using an endotoxin detection kit according to the manufacturer's instructions.

Binding of ^{125}I-MBSA-MDP with peritoneal macrophages

Plated peritoneal Mϕ were washed once with ice-cold RPMI medium containing 1 mg/mL of BSA (medium A) before starting the assay. For binding experiments, cells were incubated in 1 mL of ice-cold medium A containing different concentrations of ^{125}I-MBSA-MDP (specific activity 526 cpm/ng protein) in the presence or absence of excess MBSA (300 μg/mL) at 4°C for 3 h. Unbound material was removed by washing cells three times with chilled PBS containing 1 mg/mL BSA and once with PBS. Washed cells were dissolved in 400 μL of 0.1 N NaOH, and the cell-associated radioactivity was measured by placing an aliquot in a γ-radiation counter. Protein content of the cell lysates was estimated by the bicinchoninic acid (BCA) method [20]. Specific binding of ^{125}I-MBSA-MDP with macrophages was determined by subtracting the values for binding of ^{125}I-MBSA-MDP in the presence of excess MBSA from the total binding.

Kinetics of uptake and degradation of ^{125}I-MBSA-MDP with peritoneal macrophages

Seeded cells were washed and incubated in prewarmed medium A containing 13 μg/mL of ^{125}I-MBSA-MDP for indicated periods at 37°C. At respective time points, the amount of the TCA-soluble (non-iodide) radioactivity released in the medium was determined as degradation [21]. Cells were washed as described previously to determine the cell-associated radioactivity.

Macrophage-mediated cytolysis assay

Macrophage-mediated cytolysis of the target cells was determined by measuring [^{3}H]dThd release from prelabeled target cells [22]. The cytolytic activity of MBSA-MDP-treated J774A.1 (a murine macrophage cell line) was compared with that of free MDP-treated cells using this assay. J774A.1 cells (2×10^5) were treated with indicated concentrations of the free and conjugated MDP at 37°C in 5% CO_2 and 95% air for 24 h. Cells were washed and co-cultured with [^{3}H]dThd-prelabeled B16F10 melanoma cells at a 10:1 effector-to-target ratio for 48 h at 37°C. After incubation, the microtiter plates were centrifuged for 2 min at 250 *g*, and an aliquot of supernatant was assayed for radioactivity released from the target cells. The percent specific cytolysis was calculated using the following formula: %cytolysis = [(cpm treated wells − cpm untreated wells)/cpm of target cells added] × 100.

Macrophage-mediated inhibition of [^{3}H]thymidine incorporation by the target cells

Inhibition of [^{3}H]dThd incorporation by the target tumor cell was measured to determine the cytotoxic activity of peritoneal resident macrophages treated with free or conjugated MDP [22]. Mouse peritoneal resident macrophages (2×10^5) were incubated with indicated concentrations of the free and conjugated MDP at 37°C in 5% CO_2 and 95% air atmosphere for 24 h. Cells were washed and cocultured with L929 fibroblast as the target cell at 10:1 effector-to-target ratio for 48 h at 37°C and pulsed with 0.5 μCi [^{3}H]dThd for the last 24 h of incubation. The cells were harvested and analyzed for radioactivity incorporated. The results were expressed as percent inhibition of [^{3}H]dThd incorporation by the target cells. Percentage inhibition was calculated as: %inhibition = [(cpm of untreated well − cpm of treated well)/cpm untreated wells] × 100.

Estimation of soluble mediators released by activated macrophages

Supernatants from treated and untreated peritoneal resident macrophages were analyzed for tumor necrosis factor, interleukin (IL)-1, IL-6, IL-10, IL-12, and PGE_2, through the use of enzyme-linked immunoassays as described in the supplied kits. The ability of macrophages to secrete reactive nitrogen intermediates was also analyzed by the Greiss reaction for nitrite [23].

RESULTS

Recognition of MBSA-MDP by macrophages

The data presented in **Figure 1** show that when mouse peritoneal resident Mϕ cells were incubated with different

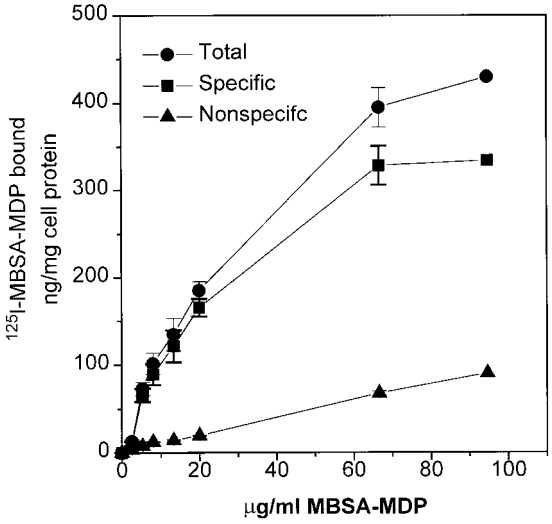

Fig. 1. Binding of MBSA-MDP to mouse peritoneal resident Mϕ at 4°C. The Mϕ (10^6) were incubated with indicated concentrations of ^{125}I-MBSA-MDP (specific activity 526 cpm/ng) for 3 h at 4°C in the absence or presence of excess MBSA (300 μg/mL) and processed as described under Materials and Methods. Specific binding was obtained by subtracting the values for nonspecific binding from the total binding. Results are expressed as nanograms labeled protein bound per milligram cellular protein and represents an average of three determinations ± SE.

concentrations of ^{125}I-MBSA-MDP for 3 h at 4°C, MBSA-MDP bound to these cells with saturation kinetics. The half-maximal binding to the cells was achieved at approximately 15 μg/mL of ^{125}I-MBSA-MDP in the medium.

When Mϕ were incubated with 13 μg/mL of ^{125}I-MBSA-MDP at 37°C for different periods of times, the cell-associated radioactivity reached a steady-state plateau after about 60 min, but trichloroacetic acid (TCA)-soluble radioactivity continued to appear in the medium, suggesting simultaneous uptake and degradation of the drug conjugate (**Fig. 2A**). The amount of radioactivity accumulated in the medium after 5 h was about three times higher than the cell-associated radioactivity. Both uptake and degradation of MBSA-MDP was competed by MBSA but not by MDP (Fig. 2B). Moreover, ^{125}I-MBSA-MDP degradation was blocked by the lysosomotropic agent, monensin (data not shown), indicating lysosomal degradation of the conjugate after internalization.

To determine whether the uptake of MBSA-MDP was mediated via the SCR, we determined the concentrations of various negatively charged compounds required to cause 50% inhibition of the degradation of ^{125}I-MBSA-MDP (13 μg/mL) by these cells. The results presented in **Table 1** show that relatively low concentrations (3–7 μg/mL) of the effective competitors, viz., MBSA, fucoidin, polyguanylic acid (PolyG), and polyinosinic acid (PolyI) brought about 50% inhibition of degradation of radiolabeled conjugate. In contrast, much higher concentrations (20-fold or more) of other negatively charged molecules, namely, polyadenylic acid (PolyA), polycytidylic acid (PolyC), and fetuin were required for inhibiting degradation to the same extent. Similarly, binding of ^{125}I-MBSA-MDP by the Mϕ were also inhibited by the SCR ligands (data not shown).

Activation of tumoricidal properties of macrophages

To determine the effect of MBSA-MDP treatment of Mϕ on their tumoricidal properties, mouse peritoneal Mϕ or J774A.1 cells were cultured in the presence of indicated concentrations of MBSA-MDP or free MDP for 24 h, washed and co-cultured with target cells at a ratio of 10:1 (effector:target) for a further 48 h. About 50% lysis of the target cells was achieved (**Fig. 3A**) when B16F10 melanoma cells prelabeled with [^{3}H]dThd were incubated with J774A.1 Mϕ treated with 1 μg/mL MBSA-MDP. Treatment of the Mϕs with free MDP, BSA-MDP, or MBSA at a dose equivalent to MBSA-MDP did not show any cytolytic activity toward the target cells. MBSA-MDP-treated peritoneal Mϕ co-cultured with B16F10 melanoma cells also exhibited similar cytolytic activity (data not shown).

To evaluate the cytotoxic efficacy of activated Mϕ using L929 fibroblasts as the target, mouse peritoneal Mϕ were treated with indicated concentrations of MBSA-MDP or free MDP for 24 h, washed, and co-cultured with L929 target cells at a target-to-effector ratio of 1:10 for another 24 h, followed by [^{3}H]dThd pulsing for an additional 24 h. The results presented in Figure 3B show that 5 μg/mL of MDP inhibited about 20% of [^{3}H]dThd uptake by target cells, but the similar effect was obtained by 0.1 μg/mL of MDP in the conjugated form under the identical condition. Moreover, the cytolytic activity of the MBSA-MDP-treated Mϕ toward L929 target cells was abolished when these cells were incubated along with PolyG, a high-affinity ligand for the SCR (**Fig. 4**).

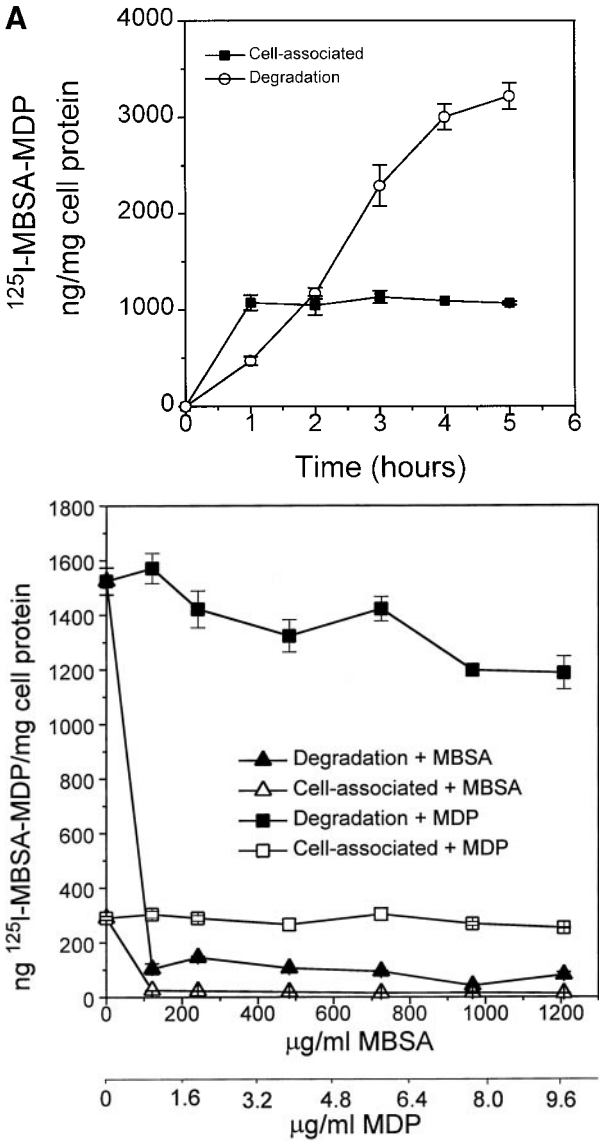

Fig. 2. (A) Time course of uptake and degradation of ^{125}I-MBSA-MDP by mouse peritoneal resident Mϕ at 37°C. Mϕ (10^6) were incubated at 37°C with 13 μg/mL of ^{125}I-MBSA-MDP as in Figure 1. At indicated time intervals, the amount of ^{125}I-MBSA-MDP accumulated in the cells and ^{125}I-labeled TCA-soluble degradation products released in the medium were determined as described in Materials and Methods. Results are the average of three determinations ± SE and expressed as nanograms labeled protein taken up or degraded per milligram cellular protein. (B) Effects of MDP and MBSA on the uptake and degradation of ^{125}I-MBSA-MDP by mouse peritoneal resident Mϕ at 37°C. Mϕ (10^6) were incubated with 13 μg/mL of ^{125}I-MBSA-MDP in presence of either MDP or MBSA at 37°C. At indicated time intervals, supernatants and cells were processed for degradation and cell-associated radioactivity, respectively. Results are expressed as nanograms labeled protein associated with the cells or degraded per milligram cellular protein and represents an average of three determinations ± SE.

Enhanced secretion of tumor-suppressive cytokines by activated macrophages

To further explore the mechanism of anti-tumor activation, we measured the levels of various soluble mediators secreted by the Mϕ after treatment with MDP. Treatment of peritoneal

TABLE 1. Degradation of ^{125}I-MBSA-MDP by Mϕ: Competition by Negatively Charged Macromolecules

Effective competitor	IC_{50}	Ineffective competitor	IC_{50}
Fucoidin	5.5	Fetuin	1290
PolyG	3.1	PolyC	109
PolyI	3.0	PolyA	64
MBSA	7.0		

Mouse peritoneal Mϕ were incubated with ^{125}I-MBSA-MDP (specific activity 526 cpm/ng) at 37°C in the presence or absence of various test samples. After 5 h, supernatants were tested for the degradation product of ^{125}I-MBSA-MDP. IC_{50}: effective concentration in μg/mL required for 50% inhibition of degradation of 15 μg/mL ^{125}I-MBSA-MDP.

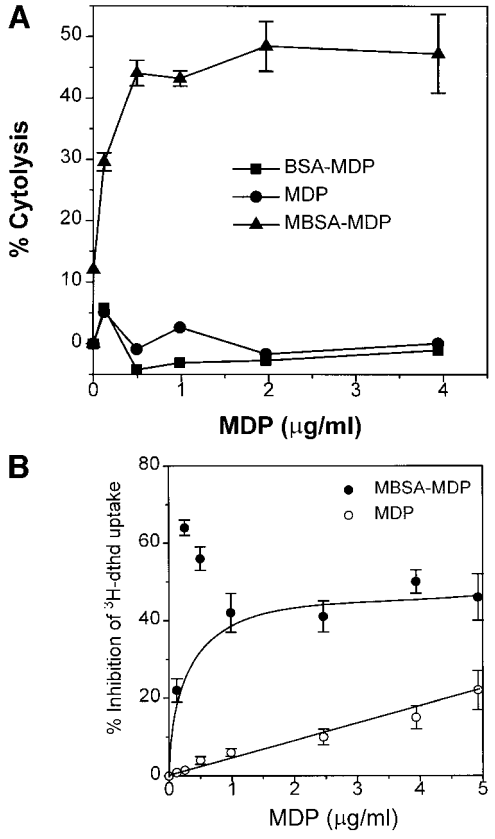

Fig. 3. (A) Effect of free MDP and MBSA-MDP on J774A.1 Mϕ-mediated B16F10 tumor cytolysis. Mϕ (2×10^5) were incubated with indicated concentrations of MDP in the free form or conjugated with BSA or MBSA. After 24 h, Mϕ were washed and target tumor cells prelabeled with [^{3}H]dThd were added at a 10:1 Mϕ-to-target cell ratio (25,400 ± 400 cpm/well). Radioactivity released in the culture medium 48 h after addition of the target cells to Mϕ was monitored. Data represented as % cytolysis are an average of three determinations ± SE. (B) Effect of MDP or MBSA-MDP treatment on anti-tumor activity of mouse peritoneal resident Mϕ. Mϕ (2×10^5) were treated with indicated concentrations of MDP in the free or conjugated form (MBSA-MDP) for 24 h. Treated macrophages were washed and target L929 cells (2×10^4) were added to the Mϕ monolayers and cocultured for 24 h. Cells were pulsed with [^{3}H]dThd (1 μCi/well) for the subsequent 24 h and radioactivity incorporated by the target cells were measured. Total [^{3}H]dThd incorporated by the target cells in the absence of macrophages was 45,690 ± 1200. Results are expressed as % growth inhibition from the average of three determinations ± SE as described in Materials and Methods.

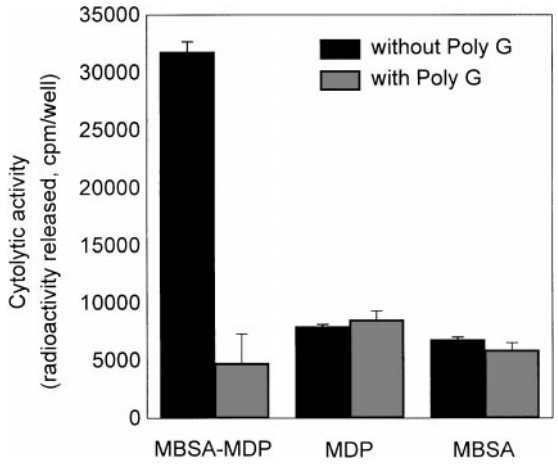

Fig. 4. Effect of PolyG on Mϕ-mediated tumor cytolytic activity. Mouse peritoneal resident Mϕ (2×10^5) were treated with 0.25 μg/mL in the free or conjugated form (MBSA-MDP) or with conjugate equivalent protein control (MBSA) for 24 h in the presence or absence of PolyG (10 μg/mL). Treated macrophages were washed, and [^{3}H]dThd prelabeled target L929 tumor cells were added at a 10:1 macrophage-to-target cell ratio (43,879 ± 978 cpm/well). Radioactivity released in the culture medium 48 h after addition of the target cells to Mϕ was monitored. Data represent an average of three determinations ± SE. Radioactivity released by the cytolytic activity of untreated macrophages was 6020 ± 202 cpm.

resident Mϕ with MBSA-MDP (equivalent to 0.25 μg/mL) triggered twofold higher levels of TNF-α and IL-6, and 10-fold higher levels of IL-1 when compared with that released on treatment with free MDP (**Fig. 5A**). Secreted levels of PGE_2 were comparable to those obtained on free MDP treatment. MBSA-treated Mϕ exhibited low levels of TNF-α and IL-6 but triggered a fivefold higher level of IL-1 compared with free MDP. Unlike MBSA-MDP treatment, MDP in the free form triggered the Mϕ suppressor cytokine, IL-10 (Fig. 5A). Significant levels of IL-12 were detected soon after MBSA-MDP treatment of Mϕ and this secretory response was detectable even 48 h after treatment. Neither MDP nor MBSA was able to induce IL-12 release in peritoneal resident Mϕ (Fig. 5A). MBSA-MDP treatment was also able to elicit sevenfold higher nitric oxide (NO) response than that triggered by MDP (**Table 2**). The enhanced secretion of cytokines by MBSA-MDP-treated macrophages was not due to the endotoxin contamination. Endotoxin contamination in MDP in free or conjugated form (equivalent to 0.25 μg/mL of MDP) was found to be less than 0.03 EU through the use of a *Limulus* lysate endotoxin detection kit (data not shown). The results presented in Figure 5B show that release of TNF-α, IL-6, IL-12, and NO is significantly inhibited when the Mϕs were treated with MBSA-MDP in the presence of excess PolyG (10 μg/mL), indicating that the SCR-mediated uptake is required for inducing secretion of these cytokines.

Role of secreted metabolites in anti-tumor activity of activated macrophages

To determine the role of NO released by Mϕ treated with MBSA-MDP, cytotoxic activity of the activated Mϕ were measured in the presence of aminoguanidine (20 μg/mL). The data presented in **Figure 6A** shows that about 50% of the

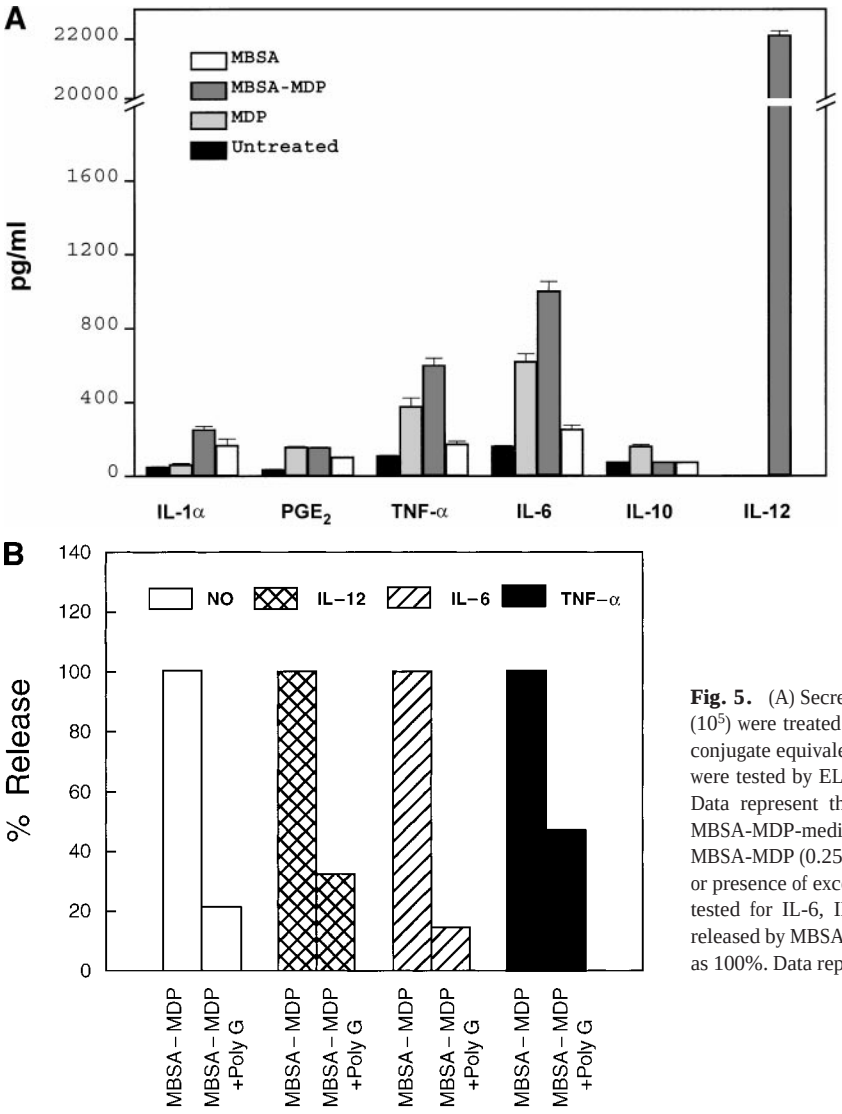

Fig. 5. (A) Secretory status of mouse peritoneal resident Mϕ on treatment. Mϕ (10^5) were treated with 0.25 μg/mL MDP in the free or conjugated form or with conjugate equivalent protein (MBSA) control. After 24-h treatment, supernatants were tested by ELISA for IL-1, IL-6, IL-10, IL-12, TNF-α, and PGE_2 released. Data represent the average of three determinations ± SE. (B) Inhibition of MBSA-MDP-mediated Mϕ activation by PolyG. Mϕ (10^5) were treated with MBSA-MDP (0.25 μg/mL MDP equivalent) in the conjugated form in the absence or presence of excess PolyG (10 μg/mL). After 24 h treatment, supernatants were tested for IL-6, IL-12, TNF-α, and NO released by the Mϕ. Each cytokine released by MBSA-MDP-treated Mϕ in the absence of excess PolyG were chosen as 100%. Data represent the average of three determinations.

anti-tumor activity is blocked by aminoguanidine, a specific inhibitor of inducible NO synthase. Similarly, antibodies against TNF-α, IL-1, or IL-6 blocked about 50% of the cytotoxic activity of Mϕ induced on MBSA-MDP treatment (Fig. 6A). Addition of anti-TNF-α along with anti-IL-1 or anti-IL-6 further reduced the cytotoxic activity of the Mϕ toward the tumor cells to 20 or 2%, respectively, whereas anti-IL-12 antibody inhibited the cytotoxic activity by about 30%. Treatment with the antibodies alone or in combination did not cause any toxic effect to either the target or the effector cells as measured by MTT assay (data not shown).

The presence of indomethacin (IM), an inhibitor of prostaglandin synthase, during activation reduced the Mϕ-mediated cytotoxicity to the target cells by about 80% (Fig. 6B). The inhibition of PGE_2 production along with neutralization of IL-1 or IL-6 could partially restore (~50%) the cytotoxic efficacy of the activated Mϕ suggests that IL-1 or IL-6 regulates prostaglandin production. Moreover, the addition of anti-TNF-α antibodies along with IM completely blocked Mϕ anti-tumor activity.

TABLE 2. Evaluation of NO Released by Free MDP and MBSA-MDP-Treated Mϕ

Concentration	Treatment	NO released (μM)
0.25 μg/mL	MDP	<2
	MBSA-MDP	47 ± 5
	MBSA	<2
0.5 μg/mL	MDP	17 ± 3
	MBSA-MDP	134 ± 7
	MBSA	<2

NO release was measured as described in Materials and Methods and results are expressed as average of three determinations ± SE.

DISCUSSION

The modulation of antitumor properties of Mϕ by different biological response modifiers is an area of active interest for cancer chemotherapy. It has been shown that antitumor properties of macrophages can be activated by interferon-γ, lipopolysaccharide, as well as MDP. Although a powerful inducer of the antitumor activity of the Mϕ, MDP is quickly eliminated from the body, thereby limiting its therapeutic utility [24–26]. Moreover, there are conflicting reports about the existence of MDP receptor on Mϕ and the efficiency of the uptake process

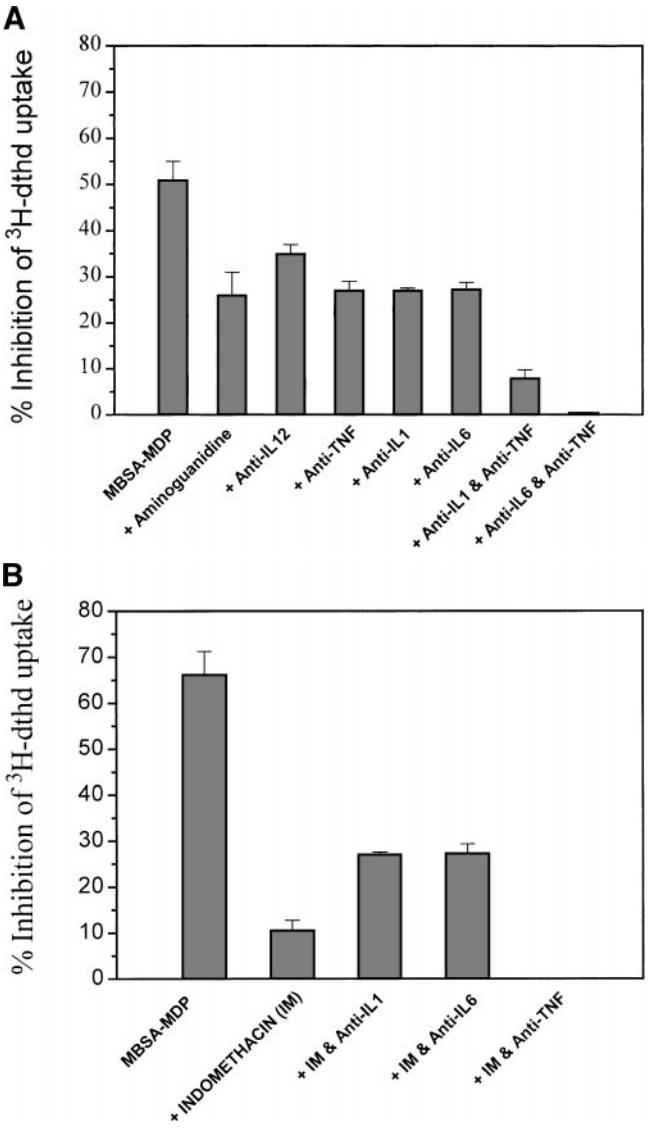

Fig. 6. (A) Effect of antibodies against interleukins on Mϕ-mediated antitumor activity induced on MBSA-MDP treatment. Mouse peritoneal resident Mϕ (2×10^5) were treated with MBSA-MDP containing 0.25 μg/mL MDP for 24 h. Treated mϕ were washed and L929 target cells (2×10^4) were added to the Mϕ monolayers along with aminoguanidine (20 μg/mL) or neutralizing antibodies to IL-1, IL-6, IL-12, or TNF (20 μg/mL) alone or in various combinations. After 24 h of co-culture, cells were pulsed with 1 μCi/well [^{3}H]dThd for 24 h and processed as described in Materials and Methods. Results are expressed as % growth inhibition and are the average of three determinations ± SE. (B) Effect of selective co-neutralization of PGE_2 and interleukins on Mϕ-mediated anti-tumor activity induced on MBSA-MDP treatment. Mouse peritoneal resident Mϕ (2×10^5) were treated with MBSA-MDP containing 0.25 μg/mL of MDP for 24 h. Treated macrophages were washed and L929 target cells (2×10^4) were added to the Mϕ monolayers along with neutralizing antibodies to IL-1, IL-6, or TNF-α (20 μg/mL) in various combinations with indomethacin (20 μg/mL). After 24 h, cells were pulsed with 1 μCi/well [^{3}H]dThd for a subsequent 24 h and processed. Mϕ alone incorporated 1128 ± 80 cpm. Total [^{3}H]dThd incorporated by the target cells in the absence of Mϕ was 30,300 ± 2052. Results are expressed as % growth inhibition from the average of three determinations ± SE.

by the Mϕ [27–29]. Accordingly, different modalities have been tried to enhance the efficacy of MDP by targeting MDP using liposomes or soluble carrier systems based on acetylated low-density lipoprotein or mannosylated neoglycoproteins [30–34]. Although targeting of MDP using some carrier increased the therapeutic efficacy of MDP, the mechanism of activation of the tumoricidal properties of Mϕ is not clear.

Because SCRs are expressed primarily on Mϕ, they have been exploited extensively for modulating Mϕ function for various purposes by specific delivery of appropriate agents [35–43]. In the present investigation, we made an attempt to specifically activate the antitumor properties of Mϕ by delivery of MDP. The results presented in these studies demonstrate that MBSA-MDP binds to discrete binding sites present on the surface of the Mϕ (Fig. 1). High-affinity binding led to rapid internalization of the MBSA-MDP followed by degradation in the lysosomes (Fig. 2A). The results presented in Figure 2B show that uptake and degradation of MBSA-MDP by Mϕ is inhibited by MBSA, suggesting that the recognition of the conjugate by Mϕ is mediated through the MBSA binding site. The data presented in Table 1 demonstrate that the degradation of MBSA-MDP is competed by the established polyanionic ligands of the SCR system, viz., MBSA, PolyG, PolyI, and fucoidin but not by other polyanionic macromolecules, e.g., PolyC, BSA-MDP, or fetuin, suggesting SCR-mediated recognition of MBSA-MDP. Although the intracellular content of MBSA-MDP was not measured in these studies, it is pertinent to mention that SCR-mediated endocytosis of the drug conjugate leads to about 8- to 10-fold higher intracellular level of the drug than that resulting from the same concentration of the free drug in the medium [14].

The MBSA-MDP-mediated activation of antitumor properties of Mϕ was monitored on two tumor cells of non-Mϕ origin, e.g., B16F10 and L929. MBSA-MDP-treated Mϕ exhibited more pronounced antitumor activity against both cells by cytotoxic and cytolytic assays than that elicited by the same concentration of the free drug. Fifty percent lysis of the target cells was achieved by 1 μg/mL of MBSA-MDP, the concentration at which the free MDP-treated Mϕ did not cause significant antitumor activity (Fig. 3A). Furthermore, when Mϕ were incubated with high concentrations of PolyG during activation with MBSA-MDP, the antitumor activity of the macrophages was totally abolished, suggesting that activation was due to the high-affinity uptake of the drug conjugate through SCR-mediated endocytosis (Fig. 4).

To understand the mechanism of macrophage activation, we measured the secretion of different cytokines and other soluble mediators as an indicator of the signal transduction cascade induced by intracellular delivery of MDP to Mϕ through SCR-mediated uptake of MBSA-MDP. The results presented in this study (Fig. 5A) show that Mϕ treated with MBSA-MDP induced several-fold higher secretion of TNF-α, IL-1, IL-6, and IL-12 compared with Mϕ treated with MBSA or free MDP. Both free MDP and MBSA-treated Mϕ, however, secreted significantly higher levels of TNF-α, IL-1, IL-6, and PGE_2 than the untreated control. These results are consistent with the observation that maleylated BSA stimulated the secretion of TNF-α [44, 45] and PGE_2 [46]. Thus, the induced secretion of different cytokines by MBSA-MDP-treated Mϕ over the free MBSA may be due to the intracellular delivery of MDP, which is supported by our results that enhanced secretion of these cytokines by MBSA-MDP-treated Mϕ is significantly inhibited in the pres-

ence of excess PolyG (Fig. 5B). The results presented in our study (Table 2) also demonstrate that MBSA-MDP-treated Mϕ induced the overproduction of NO in comparison with the cells treated with MBSA or free MDP. Recently, it has been shown that MBSA in combination with interferon-γ promote the release of NO through inducible NO synthase, but MBSA alone does not induce NO production, as also observed in our study [47]. Our results showing that cytotoxic activity of MBSA-MDP-treated Mϕ is inhibited by aminoguanidine, a specific inhibitor of inducible nitric oxide synthase [48] suggest the possibility of NO production by the activated Mϕ through the induction of inducible NO synthase. TNF-α, IL-1, IL-6, and NO are known to have tumoricidal activity [49–52] and the secretion of these molecules is also regulated by PGE_2 [53, 54]. Moreover, it was evident from our study that free MDP stimulates the secretion of IL-10, which has suppressor activity [55, 56]. In contrast, MBSA-MDP did not induce the secretion of IL-10. Thus, the antitumor activity induced by MBSA-MDP not only promotes the secretion of cytotoxic cytokines but also inhibits the secretion of cytokines that have Mϕ suppressor activity.

To determine the role of the cytokines secreted by the activated Mϕ, we measured the cytotoxic activity of MBSA-MDP-treated Mϕ in the presence of respective antibodies to each cytokine alone or in combination. The results presented in Figure 6A show that antibodies against TNF-α, IL-1, and IL-6 inhibit about 50% of the cytotoxic activity but complete inhibition was observed when anti-TNF-α antibodies were added along with anti-IL-1 or -IL-6. Similarly, inhibition of NO production by aminoguanidine also significantly reduced the cytotoxic activity of MBSA-MDP-treated Mϕ. It was, therefore, concluded that the antitumor activity induced in Mϕs on treatment with MBSA-MDP was primarily mediated through TNF-α, IL-1, IL-6, and NO. TNF-α affected macrophage effector response probably by itself and through positive regulation of IL-6 and IL-1.

PGE_2 seems to have a regulatory role in the induction of cytotoxic activity in macrophages through its role as inducer or suppressor of soluble mediators of Mϕ origin. In this investigation, we used IM, an inhibitor of prostaglandin biosynthetic pathway [57], to determine the role of prostaglandins in activation of Mϕ tumoricidal properties . Our results show that antitumor properties of the Mϕ was inhibited by IM treatment (Fig. 6B). This may be due to the down-regulation of TNF-α secretion, thereby regulating the secretion of IL-1 and IL-6 [58, 59]. These observations are further supported by the fact that simultaneous treatment of Mϕ with IM and anti-TNF-α (to neutralize residual TNF-α) totally inhibited the cytotoxic activity of the activated Mϕ, suggesting the role of TNF-α in the activation of tumoricidal activity of Mϕ. It is also possible that prostaglandins down-regulate the secretion of NO, which was shown to have antitumor activity. Moreover, inhibition of PGE_2 production along with the neutralization of IL-1 or IL-6 partially restored the cytotoxic activity of the activated Mϕ, indicating that IL-1 acts upstream of the prostaglandin synthesis pathway.

In conclusion, our data demonstrate that it is possible to augment the antitumor efficacy of Mϕ through the delivery of MDP via the SCR-mediated endocytic pathway. Intracellular delivery of MDP modulates the signal transduction pathway in Mϕ, leading to secretion of cytokines that are known to activate antitumor activity. The enhanced tumoricidal ability of Mϕ treated with MBSA-MDP is likely to be mediated by the action of TNF, IL-6 in conjunction with IL-1, and NO. We have also demonstrated that the expression of SCR as well as Mac1 remained unaltered under all the treatment conditions (data not shown). Thus, MBSA-MDP-activated Mϕ are functionally normal in terms of their SCR activity, indicating the possible use of this general modality for the treatment of cancer.

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