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Mechanism of Increased Tumor Necrosis Factor Production After Thermal Injury

Altered Sensitivity to PGE_2 and Immunomodulation with Indomethacin[1]

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ABSTRACT. Altered macrophage function after thermal injury is associated with increased production of PGE_2 and TNF. However, it is not clear why synthesis of both cellular products remains elevated, as PGE_2 is a potent inhibitor of TNF secretion. We studied the relationship between PGE_2 and TNF synthesis in a murine model of thermal injury, and examined the effect of prostaglandin blockade on splenic macrophage secretion of these mediators of inflammation. LPS-stimulated production of PGE_2 was significantly elevated in burn groups compared with sham-burned controls (pg/ml mean(SEM); sham 151(32): burn 597(147), $p < 0.01$). TNF production was similarly increased after thermal injury (pg/ml mean(SEM); sham 62(20): burn 928(316), $p < 0.01$). In vitro culture of macrophages with indomethacin augmented LPS stimulated TNF production in sham-burned controls but did not affect synthesis in burn groups, suggesting a loss of PGE_2-dependent regulation of TNF synthesis after thermal injury. Direct measurement of TNF secretion as a function of exogenous PGE_2 confirmed this dissociation between PGE_2 and TNF synthesis, as burned animals displayed a 5-fold reduction in sensitivity to PGE_2-induced inhibition of TNF, when compared with sham-burned controls (ID_{50} PGE_2 molar; sham 1.26×10^{-8}: burn 6.43×10^{-8}, $p < 0.05$). In vivo pretreatment of burn groups with indomethacin for 5 days before assay partially restored sensitivity to the prostaglandin, and significantly down-regulated synthesis of both TNF and PGE_2. These data show that thermal injury is associated with a loss of PGE_2-dependent down-regulation of TNF synthesis, which accounts at least in part for increased TNF in these animals. In vivo cyclooxygenase blockade partially restored sensitivity to the prostaglandin and consequently down-regulated synthesis of TNF. These data further support existing evidence that suggests a potential therapeutic role for cyclooxygenase blockade after major thermal injury and trauma. *Journal of Immunology,* 1993, 151: 2142.

As a result of major advances in the care of thermally injured patients over the last three decades, sepsis now ranks as one of the most important causes of mortality, accounting for 50–70% of the 10,000 burn-related mortalities in the United States each year (1–3). Major thermal injury is commonly associated with severe derangements of normal physiologic homeostasis, including cachexia, hypermetabolism, hypercortisolism and altered immune function (3–9). Although widespread defects in immune responsiveness have been reported after thermal injury, it is increasingly recognized that altered macrophage function plays a pivotal role in mediating many of the characteristic changes (10–12). In particular, persistent overproduction of PGE_2[4] and TNF has been im-

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[4] Abbreviations used in this paper: PGE_2, prostaglandin E_2; TNF, tumor necrosis factor.

plicated as a major cause of mortality and morbidity in these patients (13–18).

A number of recent studies have shown that PGE_2 plays an important inhibitory role in not only its own production, but also that of TNF (19,20). Concomitant elevation of both mediators after thermal injury suggests that their synthesis may become dysregulated. In an attempt to explain the basis for aberrant secretion of these mediators of inflammation after thermal injury, we examined the role of altered macrophage sensitivity to PGE_2 as a possible mechanism for increased production of TNF, and used these data to explore a potential therapeutic mechanism to down-regulate TNF synthesis in an experimental animal model of thermal injury.

Materials and Methods

Materials

A/J mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Indomethacin was purchased from Merck Sharpe & Dohme, West Point, PA. Ninety-six-well tissue culture plates were obtained from Nunc, Denmark. ELISA plates were obtained from Costar Corp., Cambridge, MA. All reagents for washing and cell culture were purchased from Grand Island Biological Co., Grand Island, NY. Protein concentration within wells was determined using the micro Bicinchoninic Acid (BCA) Assay from Pierce, Rockford, IL. Macrophages were stimulated with *Escherichia coli* LPS, 026:B6 from Difco, Detroit, MI. PGE_2 and the ELISA assay substrate, p-nitrophenyl phosphate disodium were obtained from Sigma Chemical Co., St. Louis, MO. The hamster anti-murine-TNF mAb, rabbit polyclonal anti-TNF antibody, and recombinant mouse TNFα were purchased from Genzyme Corp., Cambridge, MA. The alkaline phosphatase-conjugated goat anti-rabbit IgG was obtained from Boehringer Mannheim, Indianapolis, IN. PGE_2 was measured using a radioimmunoassay from Advanced Magnetics Inc., Cambridge, MA.

Animal model

Care of animals and all procedures were carried out in accordance with NIH guidelines and after review and with permission of the Harvard Medical School's Standing Committee on Animal Research. Seven-week-old male A/J mice were acclimatized for 1 wk under controlled conditions with mouse food and water *ad libitum*. Animals were randomized into burn and sham groups. A standard histologically proven 20% full thickness scald burn that was insensate was produced after inducing general anesthesia with pentobarbital (1.25 mg mouse^{-1} in 0.75 ml saline by i.p. injection). The dorsum was shaved and the burn induced by placing the animal in a plastic template that was immersed for 9 seconds in water at 90°C. Animals were resuscitated with 1 ml of saline (i.p.) after the burn. Sham burned animals underwent the same procedure, but were immersed in thermoneutral water (6).

Therapy was instituted on day 5 after injury, and consisted of the following treatment protocols: a) sham burn, saline 0.5 ml daily s.c., days 5–9, b) burn injury, saline 0.5 ml days 5 through 9, or c) burn injury, indomethacin, 0.5 μg in 0.5 ml saline, days 5 through 9.

Animals were sacrificed by placing in a CO_2 chamber on day 10 after injury. Spleens were removed and adherent cell populations prepared as outlined below. This time point was chosen as it has been shown to correspond with the period of maximum immunosuppression and susceptibility to septic challenge in this model (6).

Adherent cell preparation

Individual splenocyte suspensions were isolated and cultured as previously described (7). Adherent cells were obtained by incubating splenocyte cultures (200 μl/well at a concentration of 1×10^7/ml) in 96-well microtiter flat-bottomed plates for 1 h at 37°C and 5% CO_2 in RPMI 1640 medium, containing 2 mM/L L-glutamine, 10 mM/L HEPES buffer, and a 1% antibiotic antimycotic solution (10,000 U penicillin, 10,000 μg streptomycin, and 25 μg amphotericin B (Fungizone) per milliliter). Plates were washed three times with culture medium to remove non-adherent cells, and fresh medium was added to plates (100 μl/well). To ensure there were no differences in adherence of macrophages from each study group, the protein content of the adherent monolayer was tested. After removal of the supernatants, plates were washed with PBS and the cells lysed with 0.1 N NaOH. Protein concentration was determined using a micro Bicinchoninic Acid Assay. Our studies have shown that this method correlates well with the number of adherent cells per well (11).

Production of TNF and PGE_2, and sensitivity to PGE_2

A further 100 μl of medium containing either of the following was added to wells: a) medium only, b) LPS (final concentration 1 μg/ml), c) LPS (1 μg/ml) and indomethacin (1 μg/ml), or d) LPS (1 μg/ml) and indomethacin (1 μg/ml) and PGE_2 (10^{-6}, 10^{-7}, 10^{-8} M). Plates were incubated for a further 18 h at 37°C and 5% CO_2. Supernatants were removed and stored at -20°C until assay for PGE_2 and TNF.

Indomethacin (1 μg/ml) was added to some cultures to block de novo synthesis of PGE_2. This concentration of indomethacin was found to reduce PGE_2 synthesis to less than 5% of values obtained by stimulating with LPS alone. Dose-response curves for LPS were initially obtained to determine an appropriate submaximal stimulus for TNF synthesis (Fig. 1). Further experiments were performed to establish the optimal concentrations of PGE_2 required to achieve a 50% reduction in TNF synthesis in both sham and

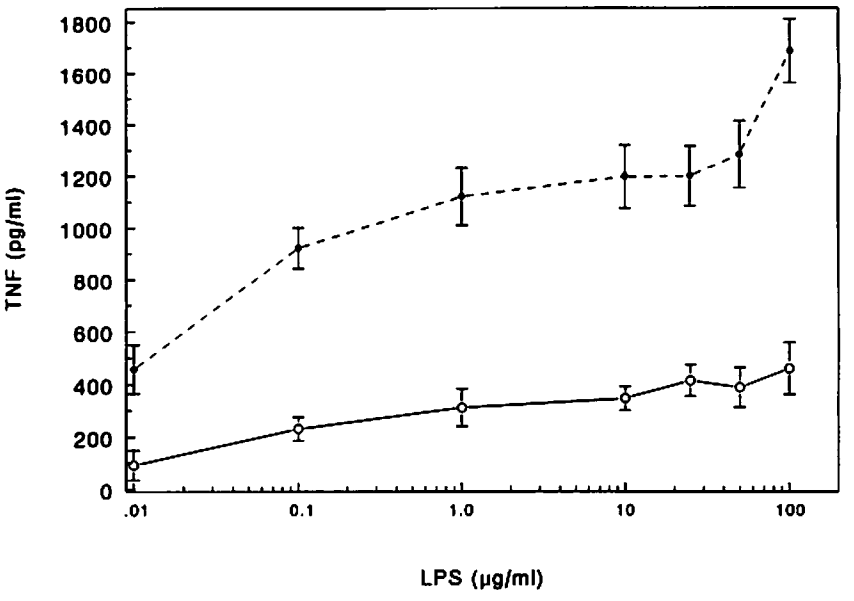

FIGURE 1. Splenic macrophage TNF synthesis in sham and burn groups as a function of stimulation with LPS (μg/ml). Assay was performed 10 days after sham (○) or burn (●) injury. Cells from each treatment group (n = 5/group) were pooled, and each point represents the mean(SEM) of three determinations. Each determination of TNF for this and all other experiments was performed in duplicate.

burn groups (i.e., 10^{-6}, 10^{-7}, 10^{-8} Molar PGE_2). Under similar culture conditions, and using the same concentration of LPS, we have previously failed to demonstrate the presence of significant quantities of retained intracellular TNF.

TNF ELISA

TNF was measured using a modified "sandwich" ELISA technique (21). Briefly, adherent cell supernatants were incubated in 96-well ELISA plates, pre-coated with hamster anti-murine-TNF mAb. Plates were washed and incubated sequentially with rabbit polyclonal anti-TNF antibody, and alkaline phosphatase-conjugated goat anti-rabbit IgG. The alkaline phosphatase linked antibody was detected at 405 nm, after incubation with the substrate p-nitrophenyl phosphate disodium. Mouse rTNFα was used as standard on each plate, and was diluted in serial twofold dilutions from 10,000 pg/ml to 9.8 pg/ml. The assay had a lower limit of sensitivity of 39 pg/ml. All determinations of TNF were carried out in duplicate.

PGE_2 assay

The concentration of PGE_2 in adherent cell supernatants was measured using a commercially available [^{3}H] radioimmunoassay, which had a lower limit of sensitivity of 80 pg/ml. Briefly, a 100 μl aliquot of the sample was incubated with 100 μl of [^{3}H]-PGE_2 tracer, and 100 μl of rabbit anti-PGE_2 antibody at 4°C for 16 h. The antibody-PGE_2 conjugate was then precipitated using a combination of dextran-coated charcoal, and centrifugation. The supernatants were decanted into scintillation vials and the β activity of the unbound [^{3}H]-PGE_2 measured in a scintillation counter. The sample concentration of PGE_2 was calculated after construction of a 6-point standard curve, which was run with each assay.

Statistical analysis

Statistical analysis was performed using the Stata® software program from the Computer Resource Center, Santa Monica, CA. The Mann Whitney two-sided test was used to compare results between groups.

Results

LPS stimulated synthesis of TNF & PGE_2 in splenic macrophages

Supernatants from adherent cell cultures, were assayed for both TNFα and PGE_2. TNF was not detected in the supernatants of cultures incubated with medium alone. Similarly, PGE_2 was not routinely detected in the absence of stimulation with LPS.

Macrophages from the burn groups produced significantly greater PGE_2 after LPS stimulation, compared with sham-burned controls (pg/ml mean(SEM); sham/saline 151(32): burn/saline 597(147), $p < 0.01$) (Fig. 2). Adherent cell TNF synthesis was also significantly increased in burn animals (pg/ml mean(SEM); sham/saline 62(20): burn/saline 928(316), $p < 0.01$) (Fig. 3).

In vivo pretreatment of burned animals with indomethacin for a 5-day period before assay significantly downregulated synthesis of TNF after thermal injury (pg/ml mean(SEM); burn/saline 928(316); burn/indomethacin 347(161), $p < 0.05$) (Fig. 3). PGE_2 was similarly downregulated after in vivo indomethacin therapy (pg/ml mean(SEM); burn/saline 597(147); burn/indomethacin 199(60), $p < 0.01$) (Fig. 2).

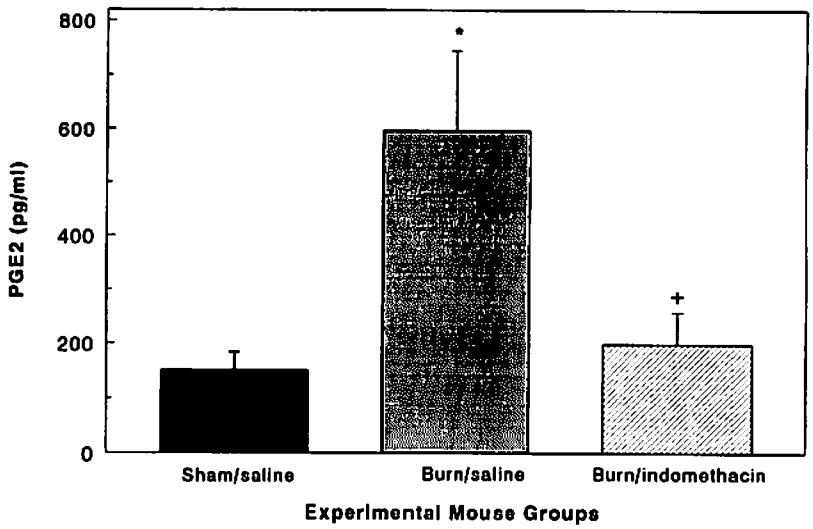

FIGURE 2. Splenic macrophage PGE_2 synthesis in treatment groups after sham or burn injury. Cultures were stimulated with LPS (1 μg/ml). Treatment was administered in vivo on days 5 through 9 to the following groups, a) sham burn; 0.5 ml saline [n = 11], b) burn injury; 0.5 ml saline, [n = 13] and c) burn injury; 0.5 μg indomethacin [n = 12]. Animals were sacrificed on day 10 post injury. PGE_2 production was assayed separately for each animal. Values represent the mean(SEM) production for each experimental group, and the data is from one of three experiments that produced similar results (* $p <$ 0.01 compared with sham/saline group; + $p <$ 0.01 compared with burn/saline group).

TNFα synthesis in the presence of in vitro cyclooxygenase blockade

Because LPS also stimulates PGE_2 synthesis, in selected experiments, indomethacin was added in vitro to cultures to examine LPS stimulated TNF synthesis, in the absence of de novo PGE_2 synthesis (Fig. 3). TNF synthesis in sham groups was increased when de novo synthesis of PGE_2 was blocked by in vitro indomethacin (pg/ml mean(SEM): LPS 62(20): LPS and indomethacin 82(32)).In contrast, TNF synthesis was marginally decreased in burn groups with the addition of in vitro indomethacin (pg/ml mean(SEM): LPS 928(316): LPS and indomethacin 863(147)).

Burn groups treated with in vivo indomethacin for a 5-day period before assay also displayed a small increase in TNF production when de novo PGE_2 synthesis was blocked by the addition of indomethacin to cultures (pg/ml mean(SEM); LPS 347(161): LPS and indomethacin 365-(60)).

Effect of burn injury on splenic macrophage sensitivity to PGE_2

The sensitivity to PGE_2-induced inhibition of TNF synthesis was directly assessed by stimulation of adherent cells in the presence of varying concentrations of PGE_2. Indomethacin was added to all cultures to block de novo synthesis of the prostaglandin.

PGE_2 inhibited TNF synthesis in a dose-dependent fashion in all treatment groups (Fig. 4). For any given concentration of PGE_2, the sham groups were significantly more sensitive to PGE_2-induced inhibition of TNF synthesis, e.g., in the presence of 10^{-8} M PGE_2, LPS-stimulated TNF production was 53% of baseline in the shams, compared with 69% in the saline-treated burn animals (p = 0.04).

Pretreatment of thermally injured animals for a 5-day period with in vivo indomethacin partially restored adherent cell sensitivity to PGE_2 (Fig. 4) and significantly reduced the concentration of PGE_2 required to produce a 50% inhibition of TNF after thermal injury, i.e., ID_{50}. The ID_{50} for the treatment groups are shown in Table I. From these data, it is apparent that a fivefold higher concentration of PGE_2 was required to halve TNF synthesis in the burn/saline group compared with sham-burned controls. Pretreatment of burn groups with indomethacin in vivo partially restored sensitivity to the prostaglandin.

Discussion

Prostaglandins and arachidonic acid metabolites have long been shown to inhibit a multitude of functions of the immune system, in both an experimental and clinical setting (22). Alterations in the synthesis of, or immune cell responsiveness to, prostaglandins and other products of arachidonic acid metabolism in various disease states has also been reported (23). PGE_2 is a potent suppressor of T-cell expression of both IL-2 (24) and IL-2 receptors (25), and also directly inhibits T-cell proliferative responses (26). Prostaglandin E_2 further interferes with monocyte-T-cell interactions by suppressing Ag presentation to T-cells, and inhibiting both Ia Ag expression and IL-1 synthesis (27–29). All of these prostaglandin-mediated defects in immune function have also been described after thermal and traumatic injury (5–10).

Similarly, excessive production of TNF may impair normal homeostasis. TNF is felt to play a pivotal role in mediating the host response to gram-negative infections and endotoxemia (30, 31) and serum TNF correlates with morbidity and mortality in sepsis and thermal injury, where increased production may precede the onset of clinical sepsis by up to 3 days (11, 14, 16). Chronic low grade secretion of TNF leads to cachexia (32), and recent evidence suggests that this cytokine may also play an important role in the

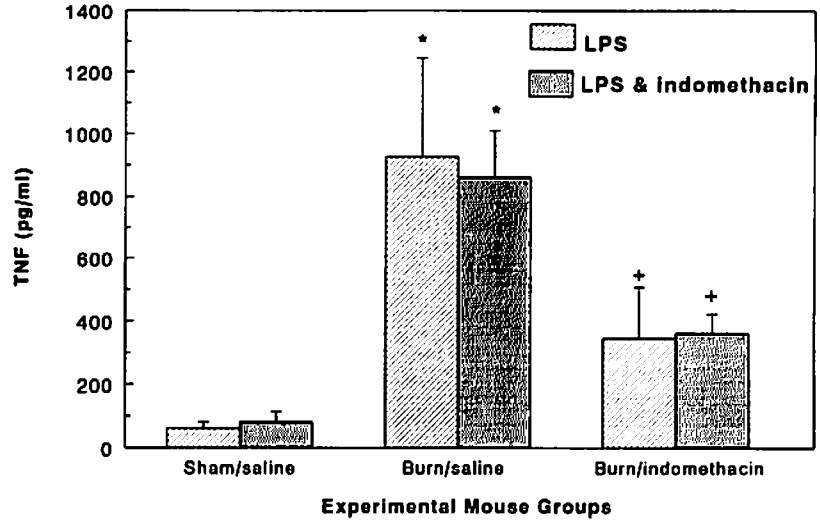

FIGURE 3. Splenic macrophage TNF production in treatment groups. Cultures were stimulated with either LPS (1 μg/ml) alone, or LPS and indomethacin (1 μg/ml). All animals were assayed individually. The values represent the mean(SEM) production in each treatment group (n = 12 per group), and the data is from one of two experiments that produced similar results. (* $p < 0.01$ compared with sham/saline group. + $p < 0.05$ compared with burn/saline group).

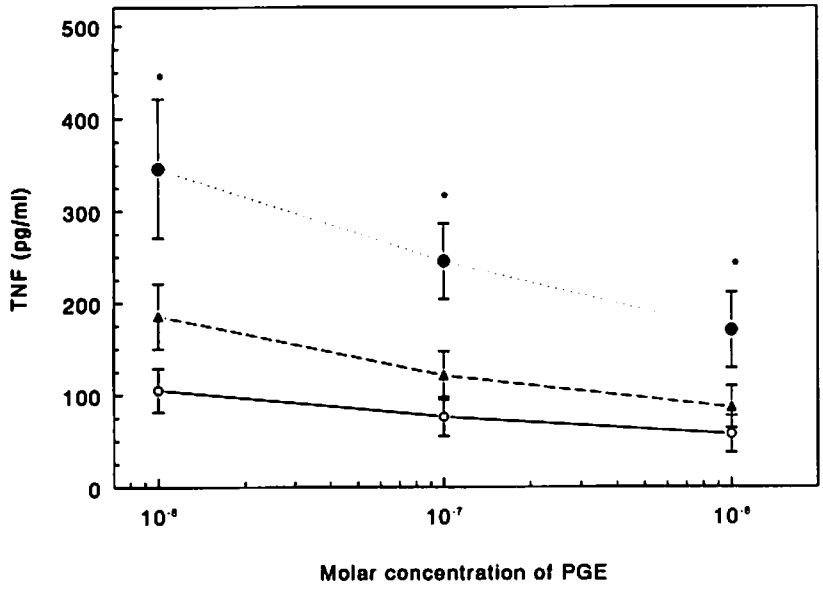

FIGURE 4. Splenic macrophage TNF production in the presence of varying concentrations of PGE_2. Cultures were stimulated with LPS and de novo synthesis of PGE_2 was inhibited by indomethacin (1 μg/ml). Treatment groups are represented by (○ sham/saline, n = 11), (● burn/saline n = 11), and (▲ burn/indomethacin n = 10). All animals were assayed individually and the data represents the mean(SEM) production for each treatment group. Data is from one of two experiments that produced similar results and from the same experiment as Table I (* $p < 0.01$ compared with sham/saline group).

characteristic hypercatabolic state that follows thermal injury (16).

Although the precise mechanisms responsible for overproduction of PGE_2 and TNF in thermal injury remain unclear, studies that have demonstrated improved immune function and survival after early burn excision and grafting suggest that the burn wound itself may play an important role in initiating defective monocyte/macrophage function (33, 34). The wound-healing process itself also induces activation of macrophages and fibroblasts, which can lead to the elaboration of high levels of prostaglandins in burn wounds (18). Major thermal injury and trauma is, however, associated with altered mononuclear phagocyte function throughout the body (35). In a recent study of monocyte function after major thermal and traumatic injury, Miller-Graziano et al. reported a progressive rise, not only in the number of monocytes producing PGE_2, but also an increase in monocyte sensitivity to agents that stimulate the release of PGE_2 (17). Although little is known of the primary events that stimulate these changes in macrophage function, hyperactivation of the mononuclear phagocyte cell population with overproduction of a range of inflammation mediators including PGE_2 and TNF appears to represent a final common pathway in the host's response to both infection and trauma (11, 14, 35).

In the present study, synthesis of both PGE_2 and TNF was significantly elevated in the burn animals compared with sham burned controls, a finding that is consistent with the observations of others (11, 13–17) (Figs. 2, 3). Sham and burn groups also behaved quite differently when cultured in the presence of in vitro indomethacin (Fig. 3). LPS-stimulated synthesis of TNF was increased in the sham groups when the inhibitory effect of de novo PGE_2 synthesis was blocked by indomethacin. These data suggest a

Table I
ID_{50} for PGE_2-induced inhibition of TNF production in splenic macrophages, and the relative sensitivities to PGE_2.

Group[a]	ID_{50}	Relative Sensitivity
Sham/saline (n = 11)	1.26×10^{-8}	1.0
Burn/saline (n = 11)	6.43×10^{-8}	0.2[b]
Burn/indomethacin (n = 10)	3.7×10^{-8}	0.34

[a] Treatment groups as for Figure 4. A separate PGE_2/TNF titration curve was constructed for each animal, and from these, the dose of PGE_2 required to produce a 50% inhibition of TNF (ID_{50}) was calculated for each animal. The data represents the mean ID_{50} for each treatment group. Data is from one of two experiments which produced similar results and from the same experiment as Figure 4.

[b] $p < 0.05$ compared to sham/saline group.

tonic inhibition of TNF synthesis by the prostaglandin and confirm previous reports that suggest that PGE_2 plays an important role in down-regulating TNF synthesis (19, 20). Macrophages from burn groups, however, displayed no significant change in TNF production with the addition of indomethacin in vitro. The failure of indomethacin to augment TNF production in this group may have been because of one of three reasons, a) PGE_2 synthesis in the burn group was not sufficiently high enough to inhibit TNF synthesis, b) the cultures were maximally stimulated, and TNF synthesis could not be further up-regulated by blocking de novo PGE_2, or c) macrophage populations from the burn group were less sensitive to PGE_2-induced inhibition of TNF production.

The first explanation is unlikely, as PGE_2 production was significantly higher in burn groups compared with sham-burned controls (Fig. 2). In addition, the dose response curve for LPS-stimulated production of TNF suggests that the concentration of LPS used in these experiments did not provide a supramaximal stimulus to macrophages from burn groups (Fig. 1). In measuring TNF production as a function of PGE_2, it is apparent that for all concentrations tested, synthesis of the cytokine was proportionately higher in the burn groups compared with sham-burned controls (Fig. 4). The concentration of PGE_2 required to produce a 50% inhibition of TNF production (ID_{50}) was five times higher after thermal injury (Table I). Pretreatment of burned animals with in vivo indomethacin for a 5-day period before assay, however restored the ID_{50} towards that of the sham-burned controls. These changes were associated with a significant reduction in synthesis of both PGE_2 and TNF (Fig. 2).

In a study of normal murine adherent cells, Kunkel et al. initially documented the importance of PGE_2 as a negative regulator for TNF gene expression (19). Further work by the same group showed that prolonged exposure to in vitro PGE_2 down-regulated macrophage sensitivity to PGE_2-induced inhibition of TNF, inducing a shift in the PGE_2/TNF curve to the right (20). Providing these cells were subsequently stimulated with LPS, TNF secretion was significantly increased, due to uncoupling of PGE_2-dependent inhibition of production. In the present study, macrophages from burn groups displayed a similar shift in the LPS/PGE_2 curve, which may be related to excessive PGE_2 synthesis in vivo (Fig. 4). In keeping with Spengler's findings, this reduction in sensitivity to PGE_2 in burn groups was also associated with increased production of TNF (Fig. 3). As a result, TNF synthesis in burn groups was unaffected by de novo PGE_2 synthesis, as evidenced by the failure of in vitro indomethacin to augment its release (Fig. 3). In contrast, the addition of indomethacin to sham burn cultures increased TNF synthesis to 134% of values obtained for stimulation with LPS alone.

Spengler et al. also noted that in vitro pretreatment of macrophages with indomethacin enhanced their sensitivity to PGE_2, leading to a down-regulation in TNF synthesis (20). Their findings, in combination with the present data, which shows that macrophages from burned mice are relatively insensitive to PGE_2, suggested a possible therapeutic mechanism to reduce overproduction of TNF, i.e., prolonged in vivo cyclooxygenase blockade which should prevent excessive PGE_2 secretion; and as suggested by Spengler's in vitro data (20), might also be expected to restore sensitivity to PGE_2-induced inhibition of TNF synthesis. Any such change would lead to an automatic down-regulation in TNF synthesis. The present study confirms this hypothesis. A 5-day course of indomethacin to burn groups led to a significant reduction in PGE_2 synthesis, which was associated with a restoration in sensitivity to the prostaglandin and decreased TNF synthesis (Table I, Fig. 2). These data suggest a potential therapeutic role to down-regulate excessive in vivo release of TNF.

It is increasingly apparent that to effectively treat severely traumatized and burned patients we must focus our attention not only on the mechanical injury, but must also attempt to elucidate the nature of the patient's own response to such events. Persistent macrophage dysfunction with overproduction of PGE_2 and TNF may be seen as one of the more important manifestations of this aberrant host response to injury. Prostaglandin blockade, either alone or in combination with other immunomodulators, is not a new concept. In vitro administration of cyclooxygenase blockers has previously been shown to restore IL-2 synthesis after major traumatic injury (15), and in vivo therapy either alone, or in combination with IL-1 and IL-2 has been reported to improve immune responses and resistance to septic challenge after thermal and traumatic injury (11, 36–38).

The present data suggest that thermal injury is associated with a major reduction in macrophage sensitivity to PGE_2, which accounts at least in part for increased synthesis of

TNF. In vivo administration of indomethacin partially restores macrophage sensitivity to the prostaglandin, and consequently down-regulates overproduction of PGE_2 and TNF after thermal injury. These data further support existing evidence that suggests a potential therapeutic role for the use of prostaglandin synthase inhibitors in severe thermal injury and trauma.

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