Prostaglandin E₂ mediates growth arrest in NFS-60 cells by down-regulating interleukin-6 receptor expression

Kumudika I. de SILVA, Asif N. DAUD, JiangPing DENG, Stephen B. JONES, Richard L. GAMELLI and Ravi SHANKAR¹

Burn and Shock Trauma Institute, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, U.S.A.

Interleukin-6 (IL-6), a potent myeloid mitogen, and the immunosuppressive prostanoid prostaglandin E₂ (PGE₂) are elevated following thermal injury and sepsis. We have previously demonstrated that bone marrow myeloid commitment shifts toward monocytopoiesis and away from granulocytopoiesis during thermal injury and sepsis and that PGE₂ plays a central role in this alteration. Here we investigated whether PGE₂ can modulate IL-6-stimulated growth in the promyelocytic cell line, NFS-60, by down-regulating IL-6 receptor (IL-6r) expression. Exposure of NFS-60 cells to PGE₂ suppressed IL-6-stimulated proliferation as well as IL-6r expression. Receptor down-regulation is functionally significant since IL-6-induced signal transduction through activators of transcription (STAT)-3 is also decreased. Down-regulation of IL-6r correlated with the ability of PGE₂ to

arrest cells in the G₀/G₁ phase of the cell cycle. PGE₂ appears to signal through EP2 receptors. Butaprost (EP2 agonist) but not sulprostone (EP3 agonist) inhibited IL-6-stimulated proliferation. In addition, an EP2 antagonist (AH6809) alleviated the anti-proliferative effects of PGE₂. NFS-60 cells express predominantly EP2 and EP4 receptors. While PGE₂ down-regulated both the IL-6r protein and mRNA expression, it had no influence on EP2 or EP4 mRNA expression. The present study demonstrates that PGE₂ is a potent down-regulator of IL-6r expression and thus may provide a mechanistic explanation for the granulocytopenia seen in thermal injury and sepsis.

Key words: bone marrow, myelopoiesis, promyelocytic cell, sepsis.

INTRODUCTION

Despite advances in critical care, sepsis and the systemic complications arising from sepsis have remained the major cause of death of critically injured patients [1,2]. Central to the pathology of sepsis is the systemic inflammatory response syndrome that is characterized by an elevation of proinflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor- α [3–6]. In particular, IL-6 levels have shown a positive correlation with increased mortality in severely injured patients, including those who have sustained massive burns [7]. Severely injured patients [8–10] and patients presenting with symptoms of sepsis have high circulating levels of IL-6. A major source of the IL-6 appears to be the monocyte/macrophage population, as evidenced by the increased IL-6 mRNA levels in peripheral blood monocytes in these patients [7]. IL-6 is a multifunctional cytokine, which promotes the proliferation and maturation of haematopoietic progenitor cells [11]. Recently it has been shown to be a major stimulator of granulopoiesis *in vivo*, independent of granulocyte colony-stimulating factor (CSF) action [12]. Despite the elevated levels of IL-6 during sepsis, profound myeloid maturation arrest resulting in neutropenia persists.

We have previously demonstrated that thermal injury superimposed with Gram-negative bacterial sepsis precipitates a critical shift in bone marrow myeloid commitment towards monocytopoiesis and away from granulocytopoiesis causing a drastic reduction in the number of circulating neutrophils [13]. We were also able to show that granulocytic maturation arrest leads to an accumulation of promyelocytes in the bone marrow [14]. Further, we demonstrated that one of the key mediators of this myeloid shift is the immunosuppressive prostanoid prostaglandin E₂ (PGE₂) [15]. PGE₂ is also a powerful inhibitor of both bone marrow myeloid and lymphoid lineages [16–23]. In addition, PGE₂ suppresses colony formation of bone marrow

cells *in vitro* [21] and when injected intravenously significantly reduces bone marrow cellularity [24]. Similar to IL-6, PGE₂ levels have also been shown to remain elevated following severe injury and sepsis both in animal models and in human patients [25–28].

We have previously shown that in burn sepsis neutropenia and granulocytopoietic maturation arrest persist despite high circulating levels of granulocyte-CSF, which is considered essential for granulocytopoietic maturation [14]. Interestingly, the inability of the myeloid cells to respond to granulocyte-CSF appears to be due to down-regulation of granulocyte-CSF receptor expression. Therefore we hypothesized that the inability of myeloid cells to respond to the elevated IL-6 levels may be due to a down-regulation of IL-6 receptor (IL-6r) expression and PGE₂ may play a critical role in this process. Here we tested this premise in the murine promyelocytic NFS-60 cell line and demonstrate that PGE₂ is indeed a powerful down-regulator of IL-6r expression and mediates its effects through an EP2-receptor-mediated cAMP signalling pathway.

EXPERIMENTAL

Cell culture

The murine promyelocytic NFS-60 cell line was cultured in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) and 10 % medium obtained from WEHI-3 cell cultures (a source of IL-3). For experimental procedures cells were suspended in RPMI 1640 medium containing 10 % FBS. Recombinant mouse IL-6 (R&D Systems, Minneapolis, MN, U.S.A.) was added at a concentration of 10 ng/ml to NFS-60 cells.

Proliferation assays

Cell proliferation was assessed based on the method of Cory et al. [29]. Cells (5×10^3) were incubated with various compounds at

Abbreviations used: Bt₂cAMP, dibutyl cAMP; CSF, colony-stimulating factor; FBS, fetal bovine serum; IL-6, interleukin-6; IL-6r, IL-6 receptor; PGE₂, prostaglandin E₂; RT-PCR, reverse transcriptase PCR; STAT, signal transduction and activators of transcription.

¹ To whom correspondence should be addressed (e-mail rshanka@bsd.lumc.edu).

37 °C in 5% CO₂ for 72 h. Inhibitors and antagonists were added 15 min prior to the addition of PGE₂. At the end of the incubation period 20 µl of a 20:1 solution of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; Promega, Madison, WI, U.S.A.] and phenazine methosulphate ('PMS'; Sigma, St. Louis, MO, U.S.A.) was added per well, incubated at 37 °C for up to 60 min and absorbance read at 495 nm.

Preparation of cell lysates

Cells were lysed in RIPA buffer (150 mM NaCl/1% Nonidet P-40/0.5% deoxycholic acid/0.1% SDS/50 mM Tris, pH 8.4) containing a protease inhibitor cocktail (Sigma). Samples were incubated at 4 °C for up to 30 min. Protein concentration in the supernatant was measured using a commercially available kit utilizing bicinchoninic acid (Pierce, Rockford, IL, U.S.A.) according to manufacturer's instructions.

Immunoprecipitation

Prior to immunoprecipitation, 1 mg of cell lysate was precleared with 0.25 µg of rabbit IgG (Accurate, Westbury, NY, U.S.A.) and Protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 30 min at 4 °C. The beads were pelleted and the supernatant was incubated with 0.8 µg of IL-6r antibody (Santa Cruz Biotechnology) for 1 h and then overnight with Protein A-agarose beads at 4 °C. Pellets were washed four times and resuspended in 30 µl of RIPA buffer containing protease inhibitors for Western blotting.

Western blotting

Equal amounts of protein from cell lysates (100 µg) or immunoprecipitated protein were mixed with Laemmli's buffer, heated at 100 °C for 5 min and resolved by SDS/PAGE (8% gel) along with prestained protein molecular-mass markers (Bio-Rad, Hercules, CA, U.S.A.). The gels were electrotransferred (15 V for 60 min) to a nitrocellulose membrane (Hybond-c extra: Amersham Biosciences, Little Chalfont, Bucks, U.K.). Membranes were incubated overnight at 4 °C with 1% casein in 11.5 mM NaH₂PO₄/10 mM Na₂HPO₄/150 mM NaCl/0.1% Tween 20/0.01% thimerosal (blocking solution), for 1 h at room temperature with IL-6r antibody (M-20 for NFS-60; Santa Cruz Biotechnology) in blocking solution and then with horseradish peroxidase-conjugated IgG (Bio-Rad) under similar conditions. Immunoblots were visualized using the enhanced chemiluminescence detection system (Pierce) following the manufacturer's instructions.

Measurement of cAMP

Cells were treated as described previously [30]. Briefly, 10⁶ cells were pretreated with 1 mM isobutylmethylxanthine ('IBMX') for 10 min at 37 °C prior to the addition of various compounds. Cells were resuspended in 500 µl of cold ethanol and cell debris removed by centrifugation. The supernatant was dried by vacuum centrifugation and resuspended in 500 µl of phosphate buffer. A cAMP enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, U.S.A.) was used to measure cAMP concentration following manufacturer's instructions.

Cell-cycle analysis

Cells were fixed in ethanol and stored at -20 °C before staining with propidium iodide. Briefly, cells resuspended in PBS were incubated sequentially in 0.1% Triton X-100 for 3 min at 4 °C,

in 180 units/ml RNase for 20 min at 37 °C and in 50 µg/ml propidium iodide for at least 1 h before analysis by flow cytometry.

Electrophoretic mobility-shift assay

Nuclear extracts were prepared from 10⁷ cells following the protocol of Schreiber et al. [31]. Nuclear protein (15 µg) was used for a gel-shift assay following standard protocols. The probe used was a double-stranded signal transduction and activators of transcription (STAT)-3 consensus sequence (Santa Cruz Biotechnology) radiolabelled with [γ -³²P]ATP (NEN, Boston, MA, U.S.A.) using T4 polynucleotide kinase (Amersham Biosciences, Piscataway, NJ, U.S.A.). The 6% polyacrylamide gel was dried under vacuum and exposed to X-ray film (Fuji, Stamford, CT, U.S.A.) for up to 24 h at -70 °C.

Reverse transcriptase PCR (RT-PCR) analysis for the expression of prostaglandin receptors

PGE receptor expression was studied by RT-PCR [32]. Total RNA from NFS-60 cells that had been treated with PGE₂ (1 µM) for 0–8 h was isolated and RT-PCR was carried out for the expression of PGE₁, PGE₂, PGE₃ and PGE₄ receptors using the following primers: EP1, sense primer, 5'-TTA ACC TGA GCC TAG CGG ATG-3', and antisense primer, 5'-CGC TGA GCG TAT TGC ACA CTA-3'; EP2, sense, 5'-GTG GCC CTG GCT CCCCC GAA AGT-3', and antisense, 5'-G GCC AAG CAT ATG GCG AAG GTG-3'; EP3- α , sense, 5'-TGA CCT TTG CCT GCA ACC TG-3', and antisense, 5'-AGC TGG AAG CAT AGT TGG TG-3'; EP3- β , antisense, 5'-GAC CCA GGG AAA CAG GTA CT-3'; EP3- γ , antisense, 5'-AGA CAA TGA GAT GGC CTG CC-3'; EP4, sense, 5'-AGT AGC TAA AGG GGG AAT CCT-3', and antisense, 5'-AAC ACT TTG GCC TGA ACT TGT-3'. β -Actin primers were sense, 5'-GTT ACC AAC TGG GAC GAC ATG G-3', and antisense, 5'-CAA AGA AAG GGT GTA AAA CGC CAG C-3'. PCR conditions for EP2 were 94 °C for 30 s, 70 °C for 1 min and 72 °C for 2 min with a final extension of 10 min at 72 °C; the total number of cycles was 30. PCR conditions for EP4 were 94 °C for 30 s, 58 °C for 1 min and 72 °C for 2 min with a final extension of 10 min at 72 °C; total number of cycles, 30. PCR conditions for β -actin were 94 °C for 30 s, 59 °C for 1 min and 72 °C for 2 min with a final extension of 10 min at 72 °C; total number of cycles, 30.

Northern analysis

Total RNA was separated from NFS-60 cells 0–6 h following treatment with PGE₂ (1 µM). RNA was separated on a formaldehyde gel and transferred to a nylon membrane. The membrane was then hybridized to a ³²P-labelled murine IL-6r cDNA and to ³²P-labelled β -actin cDNA. Murine IL-6r cDNA (GenBank accession no. X51975) was cloned from murine liver RNA by RT-PCR using the following primers: sense primer, 5'-TGATGACTGAATAGAGATGCCCG-3'; antisense primer, 5'-GCTGCTTTCCAGATGTTGAGAAGG-3'. A 244 bp product was isolated and subcloned into the pTA vector (Clontech, Palo Alto, CA, U.S.A.). The cloned cDNA was sequenced and homology with the GenBank murine IL-6r sequence was confirmed.

RESULTS

In initial experiments, using immunoblot analysis, we were able to demonstrate that PGE₂ (1 µM) was a potent down-regulator

Figure 1 Effect of PGE₂ on IL-6r expression in NFS-60 cells

NFS-60 cells were incubated in the presence and absence of 1 μ M PGE₂ for up to 8 h. Immunoprecipitation (IP) and Western blotting (WB) were carried out on cell lysates as described in the Experimental section. $n = 3$. Data represent a typical Western blot.

of IL-6r expression in the murine promyelocytic cell line NFS-60 (Figure 1). Time-course experiments showed that a significant down-regulation of IL-6r by PGE₂ became evident at 6 h and by

8 h the inhibition was almost complete. During this period the cells remained viable and did not show any evidence of either apoptosis or necrosis. To verify whether PGE₂-induced down-regulation of IL-6r in NFS-60 cells is biologically significant, we next assessed the efficacy of IL-6 to stimulate cellular proliferation and the capacity of PGE₂ to block proliferation (Figure 2). Proliferation assays corroborated the results of immunoblot analysis. IL-6 (10 ng/ml) stimulated maximal proliferation of NFS-60 cells and PGE₂ (0.01 nM–1 μ M) inhibited IL-6 induced proliferation in a dose-dependent manner. Since immunoblot analysis of IL-6r represents the status of both the cell-surface as well as the intracellular IL-6r, we wanted to verify if PGE₂ treatment was in fact capable of significantly diminishing IL-6-stimulated intracellular signals. To this end, we utilized mobility-shift assay to assess IL-6-stimulated STAT-3 activation. NFS-60 cells were incubated in the presence or absence of PGE₂ (1 μ M) for 8 h at 37 °C and then washed and exposed to IL-6 (10 ng/ml) for 15 min at 37 °C. Nuclear extracts were made from all the samples and the extent of STAT-3 activation was determined by mobility-shift assay using radiolabelled duplex oligonucleotides specific for STAT-3. Our results clearly demonstrated that IL-6 treatment was able to induce robust STAT-3 activation, as indicated by the mobility shift. Prior treatment of NFS-60 cells with PGE₂ abolished this response to a large extent (Figure 3). We were able to ascertain the specificity of the response by effective competition with unlabelled STAT-3-specific nucleotides.

Figure 2 Proliferation of NFS-60 cells

Main panel: NFS-60 cells were incubated in RPMI containing 10% FBS in the presence or absence of IL-6 (10 ng/ml) and PGE₂ (1 μ M) for 72 h. Proliferation was measured by a colorimetric assay as described in the Experimental section and is expressed as absorbance at 495 nm. Inset: dose-response curve for PGE₂. Proliferation is shown as the percentage absorbance of cells incubated in the presence of IL-6 alone (untreated control). Data represent means $\pm$ S.D. ($n = 4$).

Figure 3 Effect of PGE₂ on IL-6-induced signal transduction

NFS-60 cells were incubated in the presence or absence of 1 μ M PGE₂ for 8 h, washed and then treated with or without 10 ng/ml IL-6 for 15 min. Nuclear extracts were subjected to electrophoretic mobility-shift assay with [γ -³²P]ATP-labelled STAT-3 consensus DNA as described in the Experimental section. Excess (50 $\times$) unlabelled probe was incubated with nuclear extracts to ensure specificity of binding. $n = 3$. Data represent a typical electrophoretic mobility-shift assay.

We then investigated if the down-regulation in IL-6r protein expression is paralleled by changes in IL-6r mRNA expression. NFS-60 cells were treated with PGE₂ (1 μ M) for 0–6 h prior to RNA extraction. Northern blot analysis showed that the steady-state levels of IL-6r mRNA were reduced by 46 and 90% compared with the untreated control 2 and 4 h after PGE₂ treatment. There was no further decrease in IL-6r expression when cells were treated with PGE₂ for 8 h. Taken together, these lines of evidence support our premise that PGE₂ is indeed a powerful inhibitor of IL-6r expression in the murine promyelocytic cell line NFS-60.

Having established the potency of PGE₂ in IL-6r regulation, we queried the receptor subtype through which PGE₂ signals to accomplish its inhibitory effects. Four receptors (EP1–EP4) that mediate the effects of E-type prostaglandins have been described [33]. EP1 receptors are involved in the mobilization of intracellular calcium. EP2 and EP4 receptors have similar activity in that they stimulate adenylate cyclase but can be differentiated by distinct ligand specificity. EP3 receptor action is mainly via inhibition of adenylate cyclase although at times has been shown to signal via calcium mobilization. The potent EP1/EP3 receptor agonist sulprostone had no effect on IL-6-induced proliferation

Figure 4 Effect of sulprostone, cAMP, butaprost and AH6809 on proliferation of NFS-60 cells

NFS-60 cells were incubated in the absence or presence of 5 or 50 μ M sulprostone (black bars), Bt₂cAMP (densely hatched bars), butaprost (white bars) or AH6809 (widely hatched bars) for 72 h. Proliferation was measured as described in the Experimental section and is expressed as the percentage absorbance of cells incubated in the presence of IL-6 alone (untreated control). Data represent means $\pm$ S.D. ($n = 4$).

of NFS-60 cells (Figure 4). In contrast, the specific EP2 receptor agonist butaprost inhibited IL-6-stimulated proliferation of NFS-60 cells (Figure 4). Butaprost is not as potent as PGE₂ [33] and required a 10–50-fold higher concentration to suppress IL-6-stimulated proliferation to a similar extent as PGE₂. We also observed that the EP2 antagonist AH6809 was able to alleviate the anti-proliferative effect of PGE₂ by 30–60% ($n = 3$). In addition, exogenous cAMP [in the form of dibutyl cAMP (Bt₂cAMP)] inhibited IL-6-stimulated proliferation of NFS-60 cells (Figure 4). Taken together, these results indicate that PGE₂ may inhibit IL-6-stimulated proliferation of NFS-60 cells through its ability to generate cell signals by the EP2 receptor.

Since currently the status of the PGE receptors on NFS-60 cells remains undocumented and the response of PGE receptor expression to PGE₂ treatment is yet to be determined, we investigated the distribution and response of PGE receptors (EP1–EP4) by RT-PCR analysis in NFS-60 cells. NFS-60 cells had no detectable levels of EP1 and EP3 receptors (results not shown). However, NFS-60 cells expressed both EP2 and EP4 receptors robustly. PGE₂ treatment for 0–8 h did not significantly change the level of expression of either EP2 or EP4 receptors (Figure 5). During the same period, IL-6rs on NFS-60 cells were significantly down-regulated. Involvement of EP2/EP4 receptors in PGE₂ signalling in NFS-60 cells was further confirmed by intracellular cAMP measurements. Treatment of NFS-60 cells with 1 μ M PGE₂ increased intracellular cAMP from 17.2 ± 12.6 to 233.2 ± 75.1 pmol/10⁶ cells ($n = 3$).

PGE₂ is known to mediate its anti-proliferative effect through generation of cAMP, which leads to cell-cycle arrest at the G₁ phase [34,35]. Therefore we questioned whether, during this

Figure 5 EP2 and EP4 receptor expression in NFS-60 cells

NFS-60 cells were incubated in the presence or absence of PGE₂ (1 μ M) for 0–8 h and then total RNA was extracted as described in the Experimental section. RNA was reverse-transcribed with random primers and the first-strand cDNA was PCR amplified with primers specific for EP2, EP4 and β -actin as described in the text. The resulting products were separated on a 2% agarose gel ($n = 3$). Data represent a typical PCR amplification pattern.

Table 1 Cell-cycle distribution of NFS-60 cells following exposure to PGE₂ or cAMP

NFS-60 cells were incubated in the presence or absence of PGE₂ or Bt₂cAMP for 8 h prior to staining with propidium iodide as described in the Experimental section and analysed by flow cytometry. Results from one of three similar experiments are shown ($n = 3$). Data represent means $\pm$ S.D.

Cell-cycle phase	Cell-cycle distribution (%)			
	Untreated	1 μ M PGE ₂	5 μ M Bt ₂ cAMP	50 μ M Bt ₂ cAMP
Apoptotic	0.06 $\pm$ 0.01	0.7 $\pm$ 0.1	1.6 $\pm$ 0.15	3.9 $\pm$ 0.23
G ₀ /G ₁	45.1 $\pm$ 1.2	62.9 $\pm$ 2.1	69.7 $\pm$ 3.0	74.5 $\pm$ 2.6
S	39.8 $\pm$ 1.5	21.1 $\pm$ 1.6	15.0 $\pm$ 2.1	7.3 $\pm$ 0.9
G ₂ /M	14.9 $\pm$ 2.2	15.1 $\pm$ 1.8	13.5 $\pm$ 1.1	12.2 $\pm$ 1.3

PGE₂-mediated anti-proliferative effect on NFS-60 cells, there is any correlation between cell-cycle arrest and IL-6r down-regulation. NFS-60 cells exposed to PGE₂ or Bt₂cAMP for 8 h were stained with propidium iodide and subjected to flow-cytometric analysis. We demonstrate that exposure to PGE₂ or exogenous cAMP results in the accumulation of NFS-60 cells in the G₀/G₁ phase of the cell cycle (Table 1). Thus the reduction in IL-6r expression occurs in a similar time frame to cell-cycle arrest.

DISCUSSION

In this study we have shown that PGE₂ is a potent down-regulator of IL-6r expression in the murine promyelocytic cell line NFS-60. This inhibition is manifest in the inability of IL-6 to stimulate either proliferation or initiate intracellular signalling in the presence of PGE₂. Previous studies from our laboratory have revealed that in thermally injured animals with superimposed sepsis bone marrow myeloid commitment shifts away from granulocytopenia and toward monocytopenia [13] and this shift occurs despite elevated levels of the stimulators of granulocytopenia granulocyte-CSF and IL-6 [14,36]. Abrogation of PGE₂ production or PGE₂ receptor blockade was effective in improving survival and normalizing the myeloid commitment following burn sepsis [15,37]. In the current work, we have tried

to explore the mechanisms behind these observations through an *in vitro* cell-culture model system. While such approaches provide valuable mechanistic information, they have limitations in terms of providing a total explanation for the *in vivo* observations. Nevertheless our work clearly indicates how PGE₂ might impede the ability of IL-6 to promote myeloid progenitor cell growth.

In NFS-60 cells we observed a reduction in IL-6r expression after 6–8 h of exposure to PGE₂. We chose to monitor nuclear STAT-3 accumulation in order to determine whether this reduction in total cellular IL-6r expression was functionally significant. Within minutes of IL-6 binding to its receptor the signal-transducing moiety gp130 associates with this complex [38]. Subsequently, members of the Jak family of tyrosine kinases associated with the receptor complex become activated and phosphorylate gp130, which creates docking sites for STAT-1 and STAT-3. The STATs are phosphorylated, dimerize and are translocated into the nucleus where they associate with nuclear response elements and regulate the transcription of target genes. We demonstrated that exposure of NFS-60 cells to PGE₂ for 8 h did indeed abolish the ability to induce nuclear STAT-3 accumulation following stimulation with IL-6, indicating that the reduction in IL-6r expression was functionally significant. Thus, when elevated levels of IL-6 and PGE₂ co-exist, as in thermal injury, the effects of PGE₂ may be able to eliminate proliferative signals through the IL-6r, resulting in impaired granulocytopenia. Similar to the results we present here, PGE₂-induced cAMP inhibits human T-cell activation and proliferation by down-regulating IL-2 receptor α mRNA as well as cell-surface expression of the IL-2 receptor [16,39]. In contrast, in another *in vitro* cell system, PGE₂ has been shown to inhibit macrophage-CSF-dependent proliferation of bone marrow-derived macrophages without affecting expression of the CSF-1 receptor or early signal transduction mediated by ligand binding [34,40]. A potential explanation for the lack of effect on CSF-1 receptor expression is perhaps due to the time frame in the CSF-1 studies. These investigators examined the very early effects of PGE₂ (1 h), whereas in our study and in studies involving T-cell proliferation and IL-2 receptor expression much longer-term effects (8–24 h) were studied.

We confirm previous reports [34,35] that the anti-proliferative effects of PGE₂ are mediated by cAMP (Figures 2 and 4) and show for the first time that in NFS-60 cells these effects are mediated through the EP2 receptor. EP2 receptors are associated with adenylate cyclase and mediate signal transduction by increasing intracellular cAMP levels [33]. We demonstrated that the EP2-receptor-specific agonist butaprost inhibits IL-6-induced proliferation of NFS-60 cells. In addition, the EP2 receptor antagonist AH6809 relieves PGE₂-induced inhibition of IL-6-stimulated proliferation of these cells.

Previous studies have established that elevating cAMP levels inhibit mitogen-dependent DNA synthesis and proliferation of myeloid cells [34,35]. cAMP also halts progression of the cell cycle by down-regulating D-type cyclins and cyclin-dependent kinase 4 (cdk4) [41]. Other anti-proliferative effects of cAMP include suppressing phosphorylation of retinoblastoma protein and expression of c-Myc and proliferating cell nuclear antigen [35]. There is evidence that similar effects may occur following thermal injury. In an animal model intracellular cAMP levels are elevated in splenocytes following thermal injury and cAMP suppresses mitogen-stimulated proliferation of normal splenocytes [42].

The PGE₂-induced decrease in IL-6r expression coincides with the accumulation of NFS-60 cells in the G₀/G₁ phase of the cell cycle and our studies with exogenous Bt₂cAMP demonstrate that cAMP is the secondary signal which mediates this cell-cycle

arrest (Table 1). These results are consistent with similar findings in the current literature. For example, raising intracellular cAMP by either exogenous addition or stimulation with PGE₂ has been shown to arrest macrophage growth by arresting the cells in the mid-G₁ phase of the cell cycle [43]. Similar results were also reported in airway smooth muscle cells [44]. Nonetheless, no study until now has shown both PGE₂- and cAMP-mediated cell-cycle arrest and receptor down-regulation in the same cell type. However, it is not known whether the down-regulation of the IL-6 α and cell-cycle arrest are related or are independent events initiated by PGE₂.

Our studies also provide the PGE₂ receptor distribution in NFS-60 cells. These cells predominantly express EP2 and EP4 receptors. Exposure to PGE₂ for 0–8 h did not significantly down-regulate EP2 and EP4 receptors. Recent studies on mouse calvaria have demonstrated that PGE₂ causes bone resorption through cAMP generated by the engagement of EP2 and EP4 receptors [45,46]. Furthermore, using knockout mice specific for each type of EP receptor, Nataraj et al. [32] have demonstrated that the anti-proliferative effect is independent of the EP1 and EP3 receptors. They further showed that PGE₂ activated EP4 receptors on macrophages. Similar agonist-induced up-regulation of EP4 receptor expression in macrophages has also been demonstrated by Hubbard et al. [47]. Our studies, however, demonstrate that EP receptor expression in NFS-60 cells remains unchanged for the duration of the experiment. PGE₂ appears, however, to down-regulate IL-6 α s during the similar time period at both the protein and mRNA levels and this results in inhibition of the IL-6-mediated intracellular signalling.

In summary, the current study demonstrates that PGE₂ is a potent down-regulator of IL-6 α expression in NFS-60 cells and is likely to mediate this effect through either EP2 and/or EP4 receptor activation. Furthermore, the current study provides a potential mechanism for the inhibition of granulocytopenia and the resulting neutropenia in animals with burn injury and sepsis even in the presence of elevated levels of myeloid mitogens such as IL-6.

This work was supported by National Institutes of Health grants R01 GM56424 (to R.S.) and R01 GM42577 (to R.L.G.). We thank Patricia Simms for flow-cytometric data collection and analysis.

REFERENCES

- Chaudry, I. H. (1999) Sepsis: lessons learned in the last century and future directions. *Arch. Surg.* **134**, 922–929.
- Gullo, A. (1999) Sepsis and organ dysfunction/failure. An overview. *Minerva Anesthesiol.* **65**, 529–540.
- Bone, R. C. (1994) Sepsis and its complications: the clinical problem. *Crit. Care Med.* **22**, S8–S11.
- van der Poll, T. and van Deventer, S. J. (1999) Cytokines and anticytokines in the pathogenesis of sepsis. *Infect. Dis. Clin. North Am.* **13**, 413–426.
- Terregino, C. A., Lopez, B. L., Karras, D. J., Killian, A. J. and Arnold, G. K. (2000) Endogenous mediators in emergency department patients with presumed sepsis: are levels associated with progression to severe sepsis and death? *Ann. Emerg. Med.* **35**, 26–34.
- Presterl, E., Staudinger, T., Pettermann, M., Lassnigg, A., Burgmann, H., Winkler, S., Frass, M. and Graninger, W. (1997) Cytokine profile and correlation to the APACHE III and MPM II scores in patients with sepsis. *Am. J. Respir. Crit. Care Med.* **156**, 825–832.
- Schluter, B., König, B., Bergmann, U., Müller, F. E. and König, W. (1991) Interleukin 6 – a potential mediator of lethal sepsis after major thermal trauma: evidence for increased IL-6 production by peripheral blood mononuclear cells. *J. Trauma* **31**, 1663–1670.
- Guo, Y., Dickerson, C., Chrest, F. J., Adler, W. H., Munster, A. M. and Winchurch, R. A. (1990) Increased levels of circulating interleukin 6 in burn patients. *Clin. Immunol. Immunopathol.* **54**, 361–371.
- Ueyama, M., Maruyama, I., Osame, M. and Sawada, Y. (1992) Marked increase in plasma interleukin-6 in burn patients. *J. Lab. Clin. Med.* **120**, 693–698.
- Nijsten, M. W., Hack, C. E., Helle, M., ten Duis, H. J., Klasen, H. J. and Aarden, L. A. (1991) Interleukin-6 and its relation to the humoral immune response and clinical parameters in burned patients. *Surgery* **109**, 761–767.
- Kishimoto, T. (1989) The biology of interleukin-6. *Blood* **74**, 1–10.
- Liu, F., Poursine-Laurent, J., Wu, H. Y. and Link, D. C. (1997) Interleukin-6 and the granulocyte colony-stimulating factor receptor are major independent regulators of granulopoiesis *in vivo* but are not required for lineage commitment or terminal differentiation. *Blood* **90**, 2583–2590.
- Santangelo, S., Gamelli, R. L. and Shankar, R. (2001) Myeloid commitment shifts toward monocytopenia after thermal injury and sepsis. *Ann. Surg.* **233**, 97–106.
- Shoup, M., Weisenberger, J. M., Wang, J. L., Pyle, J. M., Gamelli, R. L. and Shankar, R. (1998) Mechanisms of neutropenia involving myeloid maturation arrest in burn sepsis. *Ann. Surg.* **228**, 112–122.
- Santangelo, S., Shoup, M., Gamelli, R. L. and Shankar, R. (2000) Prostaglandin E2 receptor antagonist (SC-19220) treatment restores the balance to bone marrow myelopoiesis after burn sepsis. *J. Trauma* **48**, 826–830; discussion 830–831.
- Anastassiou, E. D., Paliogianni, F., Balow, J. P., Yamada, H. and Boumpas, D. T. (1992) Prostaglandin E2 and other cyclic AMP-elevating agents modulate IL-2 and IL-2R α gene expression at multiple levels. *J. Immunol.* **148**, 2845–2852.
- Brown, D. M. and Phipps, R. P. (1997) Prostaglandin E2 mediated apoptosis in subsets of malignant B lymphoma cells. *Adv. Exp. Med. Biol.* **400B**, 565–570.
- Goodwin, J. S., Bankhurst, A. D. and Messner, R. P. (1977) Suppression of human T-cell mitogenesis by prostaglandin. Existence of a prostaglandin-producing suppressor cell. *J. Exp. Med.* **146**, 1719–1734.
- Hoshi, S., Furutani-Seiki, M., Seto, M., Tada, T. and Asano, Y. (1994) Prevention of TCR-mediated apoptosis by the elevation of cAMP. *Int. Immunol.* **6**, 1081–1089.
- Gurlo, T., Huang, W. W. and von Grafenstein, H. (1998) PGE2 inhibits IL-2 and IL-4-dependent proliferation of CTL-2 and HT2 cells. *Cytokine* **10**, 265–274.
- Kurland, J. and Moore, M. A. (1977) Modulation of hemopoiesis by prostaglandins. *Exp. Hematol.* **5**, 357–373.
- Pelus, L. M. and Gentile, P. S. (1988) *In vivo* modulation of myelopoiesis by prostaglandin E2. III. Induction of suppressor cells in marrow and spleen capable of mediating inhibition of CFU-GM proliferation. *Blood* **71**, 1633–1640.
- Pelus, L. M., Broxmeyer, H. E., Kurland, J. I. and Moore, M. A. (1979) Regulation of macrophage and granulocyte proliferation. Specificities of prostaglandin E and lactoferrin. *J. Exp. Med.* **150**, 277–292.
- Gentile, P., Byer, D. and Pelus, L. M. (1983) *In vivo* modulation of murine myelopoiesis following intravenous administration of prostaglandin E2. *Blood* **62**, 1100–1107.
- Takayama, T. K., Miller, C. and Szabo, G. (1990) Elevated tumor necrosis factor α production concomitant to elevated prostaglandin E2 production by trauma patients' monocytes. *Arch. Surg.* **125**, 29–35.
- Ninmann, J. L. and Stockland, A. E. (1984) Participation of prostaglandin E in immunosuppression following thermal injury. *J. Trauma* **24**, 201–207.
- Fukushima, R., Alexander, J. W., Wu, J. Z., Mao, J. X., Szczur, K., Stephens, A. M., Ogle, J. D. and Ogle, C. K. (1994) Time course of production of cytokines and prostaglandin E2 by macrophages isolated after thermal injury and bacterial translocation. *Circ. Shock* **42**, 154–162.
- Huribal, M., Cunningham, M. E., D'Aiuto, M. L., Pleban, W. E. and McMillen, M. A. (1995) Endothelin-1 and prostaglandin E2 levels increase in patients with burns. *J. Am. Coll. Surg.* **180**, 318–322.
- Cory, A. H., Owen, T. C., Barltrop, J. A. and Cory, J. G. (1991) Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun.* **3**, 207–212.
- Zeng, L., An, S. and Goetzl, E. J. (1996) Regulation of expression of matrix metalloproteinase-9 in early human T cells of the HSB.2 cultured line by the EP3 subtype of prostaglandin E2 receptor. *J. Biol. Chem.* **271**, 27744–27750.
- Schreiber, E., Matthias, P., Müller, M. M. and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **17**, 6419.
- Nataraj, C., Thomas, D. W., Tilley, S. L., Nguyen, M. T., Mannon, R., Koller, B. H. and Coffman, T. M. (2001) Receptors for prostaglandin E2 that regulate cellular immune responses in the mouse. *J. Clin. Invest.* **108**, 1229–1235.
- Negishi, M., Sugimoto, Y. and Ichikawa, A. (1995) Molecular mechanisms of diverse actions of prostanoid receptors. *Biochim. Biophys. Acta* **1259**, 109–119.
- Vairo, G., Argyriou, S., Bordun, A. M., Whitty, G. and Hamilton, J. A. (1990) Inhibition of the signalling pathways for macrophage proliferation by cyclic AMP. Lack of effect on early responses to colony stimulating factor-1. *J. Biol. Chem.* **265**, 2692–2701.
- Ward, A. C., Hoffmann, B. W., Csar, X. F. and Hamilton, J. A. (1996) Granulocyte colony-stimulating factor-stimulated proliferation of myeloid cells: mode of cell cycle control by a range of inhibitors. *J. Interferon Cytokine Res.* **16**, 869–877.
- Struzyna, J., Pojda, Z., Braun, B., Chomiczka, M., Sobiczewska, E. and Wrembel, J. (1995) Serum cytokine levels (IL-4, IL-6, IL-8, G-CSF, GM-CSF) in burned patients. *Burns* **21**, 437–440.

- 37 Shoup, M., He, L. K., Liu, H., Shankar, R. and Gamelli, R. (1998) Cyclooxygenase-2 inhibitor NS-398 improves survival and restores leukocyte counts in burn infection. *J. Trauma* **45**, 215–220; discussion 220–221
- 38 Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F. and Graeve, L. (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.* **334**, 297–314
- 39 Rincon, M., Tugores, A., Lopez-Rivas, A., Silva, A., Alonso, M., De Landazuri, M. O. and Lopez-Bolet, M. (1988) Prostaglandin E₂ and the increase of intracellular cAMP inhibit the expression of interleukin 2 receptors in human T cells. *Eur. J. Immunol.* **18**, 1791–1796
- 40 Hume, D. A., Allan, W., Fabrus, B., Weidemann, M. J., Hapel, A. J. and Bartelmez, S. (1987) Regulation of proliferation of bone marrow-derived macrophages. *Lymphokine Res.* **6**, 127–139
- 41 Ward, A. C., Csar, X. F., Hoffmann, B. W. and Hamilton, J. A. (1996) Cyclic AMP inhibits expression of D-type cyclins and cdk4 and induces p27Kip1 in G-CSF-treated NFS-60 cells. *Biochem. Biophys. Res. Commun.* **224**, 10–16
- 42 Horgan, A. F., O'Riordain, D. S., Chin, D. H., Mannick, J. A. and Rodrick, M. L. (1994) The role of cyclic adenosine monophosphate in the suppression of cellular immunity after thermal injury. *Arch. Surg.* **129**, 1284–1289
- 43 Rock, C. O., Cleveland, J. L. and Jackowski, S. (1992) Macrophage growth arrest by cyclic AMP defines a distinct checkpoint in the mid-G₁ stage of the cell cycle and overrides constitutive c-myc expression. *Mol. Cell. Biol.* **12**, 2351–2358
- 44 Stewart, A. G., Harris, T., Fernandes, D. J., Schachte, L. C., Koutsoubos, V., Guida, E., Ravenhall, C. E., Vadeloo, P. and Wilson, J. W. (1999) Beta2-adrenergic receptor agonists and cAMP arrest human cultured airway smooth muscle cells in the G(1) phase of the cell cycle: role of proteasome degradation of cyclin D1. *Mol. Pharmacol.* **56**, 1079–1086
- 45 Suzawa, T., Miyaura, C., Inada, M., Maruyama, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., Narumiya, S. and Suda, T. (2000) The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology* **141**, 1554–1559
- 46 Miyaura, C., Inada, M., Suzawa, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., Narumiya, S. and Suda, T. (2000) Impaired bone resorption to prostaglandin E₂ in prostaglandin E receptor EP4-knockout mice. *J. Biol. Chem.* **275**, 19819–19823
- 47 Hubbard, N. E., Lee, S., Lim, D. and Erickson, K. L. (2001) Differential mRNA expression of prostaglandin receptor subtypes in macrophage activation. *Prostaglandins Leukot. Essent. Fatty Acids* **65**, 287–294

Received 27 September 2002/7 November 2002; accepted 12 November 2002

Published as BJ Immediate Publication 12 November 2002, DOI 10.1042/BJ20021512