Cyclooxygenase-2 Regulates Granulocyte-Macrophage Colony–Stimulating Factor, but Not Interleukin-8, Production by Human Vascular Cells

Role of cAMP

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Abstract—Vascular smooth muscle is now recognized as an important site of mediator generation under inflammatory conditions. Indeed, the release of leukocyte activators, such as granulocyte-macrophage colony–stimulating factor (GM-CSF) and interleukin (IL)-8, by human arterial smooth muscle cells has recently been demonstrated. However, the potential for venous cells to release GM-CSF has not been addressed. We have shown that human vascular smooth muscle cells express the “inflammatory” form of cyclooxygenase (COX), cyclooxygenase-2 (COX-2), when stimulated with cytokines. In some nonvascular cell types, the COX activity has been shown to regulate the release of GM-CSF and IL-8, although the nature of the isoform responsible was not addressed. We show that human venous smooth muscle cells, like their arterial counterparts, release GM-CSF after stimulation with IL-1β. Similarly, both cell types released IL-8. Under the same conditions, we found that COX-2 activity suppressed GM-CSF, but not IL-8, release by both types of human vascular cells. Moreover, the prostacyclin mimetic, cicaprost, and the cAMP analogue, dibutyryl cAMP, inhibited GM-CSF release from these cells. These observations suggest that COX-2 activity suppresses GM-CSF release via a cAMP-dependent pathway in human vascular cells and illustrates a novel mechanism by which this enzyme can modulate immune and inflammatory events. (Arterioscler Thromb Vasc Biol. 2000;20:677-682.)

Key Words: inflammation ▪ neutrophils ▪ aspirin ▪ interleukin-8 ▪ atherosclerosis

In healthy blood vessels, the luminal surface is lined by a continuous monolayer of endothelial cells. The vascular endothelium, by expression of surface molecules and release of biologically active mediators, regulates vascular tone and function. In vascular diseases, such as atherosclerosis and septic shock, damage to the vessel wall results in endothelial cell disruption. This leads to the recruitment and activation of inflammatory cells, and within the vessel wall, exacerbating and propagating the inflammatory response. Neutrophils are the first inflammatory cells to appear at the site of vessel damage.

Interleukin (IL)-8 is a CXC chemokine produced by a variety of cell types, including endothelial cells, monocytes, and fibroblasts. It is a powerful and specific neutrophil chemoattractant and, for this reason, is likely to be important in the initiation and propagation of vascular disease. Once neutrophils are present at the site of inflammation, they do not differentiate and rapidly die. Granulocyte-macrophage colony–stimulating factor (GM-CSF), released from a wide range of cells, is one of a number of colony-stimulating factors responsible for the proliferation and differentiation of cells in the bone marrow. This cytokine also modulates the function of circulating mature leukocytes, including neutrophils. GM-CSF promotes activation, surface receptor expression, and survival of mature neutrophils and, therefore, may be implicated, like IL-8, in the pathogenesis of vascular disease and also have an established role as an immunomodulator.

Loss of or damage to the endothelium results in the exposure of underlying smooth muscle cells. These cells represent the major cell type in the media of normal human arteries and veins and, therefore, are potentially an important source of inflammatory mediators. Their ability to release such mediators, under inflammatory conditions, is currently the subject of investigation. Indeed, we have recently demonstrated the expression of the inducible inflammatory form of cyclooxygenase (COX), cyclooxygenase-2 (COX-2), and the subsequent release of prostaglandins from the smooth muscle component of human vessels stimulated in vitro with inflammatory cytokines. Similarly, we have demonstrated that the expression of COX-2 in human cultured venous and arterial smooth muscle cells is under the control of IL-1β and tumor necrosis factor (TNF)-α. As well as stimulating the expression of COX-2, these inflammatory cytokines can also
induce the release of IL-8 from human cultured vascular smooth muscle cells and GM-CSF from human cultured vascular and airway smooth muscle. Therefore, our aim was to use human vascular smooth muscle cells cultured from saphenous vein (SV) and internal mammary artery to investigate the relative abilities of arterial and venous cells to release IL-8 and GM-CSF. Furthermore, we have investigated the effect of coinoculated COX-2 on the release of these 2 cytokines.

Methods

Materials
Human recombinant IL-1β, TNF-α, and interferon-γ (IFN-γ) were bought from R & D Systems, and lipopolysaccharide (LPS) was obtained from Sigma Chemical Co. Trinitiated prostaglandin (PG) E2 was purchased from Amersham. Matched ELISA reagents to develop immunoassays for human GM-CSF were bought from Pharmingen. Matched IL-8 antibody pairs and human recombinant IL-8 required for human IL-8 ELISA were bought from R & D Systems. For cell culture, Hanks’ balanced salt solution (HBSS) and DMEM were purchased from Sigma, and MEM nonessential amino acids were obtained from Gibco-BRL. L-745,337 was a gift from Merck, Harlow, UK and cicaprost was a gift from Dr F. McDonald at Schering, Berlin, Germany. Nimesulide was from Sigma, and meloxicam was from Boehringer-Ingelheim. All other reagents were from Sigma.

Patients
Samples of internal mammary artery and SV, surplus to clinical requirements, were obtained from patients undergoing coronary artery bypass surgery at the Royal Brompton and Harefield N.H.S. Trust. Vessels were used regardless of patient history and preoperative drug therapy. Ethical permission was obtained from the Royal Brompton and Harefield N.H.S. Trust ethics committee. In total, SV was obtained from 8 different patients, and internal mammary artery was obtained from 7 patients.

Statistics
All data are given as mean±SEM. Statistical significance was calculated by 2-way ANOVA, 1-way ANOVA (Dunnett post hoc test), or 1-sample t test. A value of P<0.05 was taken as significant.

Cell Culture
Vessels received in sterile pots direct from surgery were prepared immediately under sterile culture conditions. Vessels were placed in Petri dishes containing sterile HBSS supplemented with penicillin (100 U/mL), streptomycin (0.1 mg/mL), L-glutamine (2.0×10⁻³ mol/L), and amphotericin B (1.0 μg/mL). Vessels were dissected clear of connective tissue during the course of repeated washings in supplemented HBSS. The vessels were then dissected along their length, and the endothelium was carefully scraped off with a scalpel blade. Vessels were cut into small pieces and placed in a 75-cm² culture flask with DMEM containing sodium pyruvate (110 mg/L) and phenol red and supplemented with penicillin, streptomycin, glutamine, amphotericin B, MEM nonessential amino acids, and 20% FCS.

After an initial 7-day period to allow the tissue pieces to adhere to the bottom of the flask, explants received new supplemented DMEM every 3 or 4 days. After ~14 days, smooth muscle cells were seen as they began to grow out from the edges of the tissue. At this point, the concentration of FCS in the supplemented DMEM was reduced from 20% to 10%. After a period of 6 to 10 weeks, cells were fully confluent. Adherent tissue pieces were then removed, and cells were passaged with trypsin in supplemented HBSS. All experiments were performed with cells (passages 2 to 6 only) from 2 to 3 different patients. For use in experiments, cells were plated onto 96-well plates. When the cells reached confluence, the phenotype of cells was synchronized to “contractile” by withdrawing serum for 24 hours before treatment with inflammatory cytokines and drugs.

Cell Treatment
At the beginning of each experiment, new supplemented DMEM (10% FCS) was added to the cells. Cells were stimulated for 24 hours with increasing concentrations of IL-1β (1.0 pg/mL to 10 ng/mL), TNF-α (10 pg/mL to 10 ng/mL), IFN-γ (1.0 to 100 ng/mL), and LPS (1.0 to 100 μg/mL). In some experiments, vascular cells were pretreated (~5 minutes) with different NSAIDs, including indomethacin (1×10⁻¹⁰ to 1×10⁻⁵ mol/L), aspirin (1×10⁻⁷ to 1×10⁻⁴ mol/L), nimesulide (1×10⁻⁷ to 1×10⁻⁵ mol/L), meloxicam (1×10⁻⁷ to 1×10⁻⁵ mol/L), and L-745,337 (1×10⁻⁸ to 1×10⁻⁵ mol/L) before the addition of either DMEM alone or DMEM containing IL-1β (1 ng/mL) for 24 hours. A further set of experiments was carried out in which cells were treated for 24 hours with increasing concentrations of PGE₂ (1×10⁻⁴ to 1×10⁻⁵ mol/L), the prostacyclin (PGI₂) mimic cicaprost (1×10⁻⁷ to 1×10⁻⁵ mol/L), or dibutyryl cAMP (1×10⁻⁴ to 1×10⁻⁵ mol/L) in the presence or absence of IL-1β (1 ng/mL) with or without indomethacin (1×10⁻³ mol/L).

IL-1β, TNF-α, IFN-γ, LPS, cicaprost, and dibutyryl cAMP were dissolved in DMEM. PGE₂ was initially dissolved in ethanol with all subsequent dilutions in DMEM (0.1% ethanol, final concentration on cells). Indomethacin, aspirin, meloxicam, nimesulide, and L-745,337 were all dissolved initially in dimethyl sulfoxide (DMSO) with all subsequent dilutions in DMEM (final concentration on cells: aspirin 1% DMSO; indomethacin, meloxicam, nimesulide, and L-745,337 0.1% DMSO). In the concentrations present, neither DMSO nor ethanol affected the release of PGE₂ and GM-CSF or cell viability as assessed by mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to formazan.

PGE₂ was measured by radioimmunoassay with the use of commercially available tritiated PGE₂, as previously described. GM-CSF and IL-8 were measured by ELISA established in-house from individual constituents according to the manufacturer’s recommendations.

Results

Release of PGE₂, GM-CSF, and IL-8 From Human Cultured Arterial and Venous Smooth Muscle Cells
Human arterial smooth muscle cells spontaneously released low levels of PGE₂ (7.7±1.1 ng/mL, n=41), GM-CSF (50.0±15.2 pg/mL, n=48), and IL-8 (5.7±0.3 ng/mL, n=9) over a 24-hour period under control culture conditions. Venous smooth muscle cells also spontaneously released PGE₂ (18.4±2.9 ng/mL, n=45) and IL-8 (9.9±1.6 ng/mL, n=24) under these conditions. However, levels of GM-CSF released by venous cells were so low as to be undetectable by the ELISA protocol used (ie, <15.6 pg/mL, n=78).

When arterial cells were stimulated for 24 hours with either IL-1β (1.0 pg/mL to 10 ng/mL) or TNF-α (10 pg/mL to 10 ng/mL), a concentration-dependent increase in PGE₂, GM-CSF, and IL-8 release was observed (Figure 1). Similarly, when venous cells were stimulated with IL-1β, there was a concentration-dependent increase in PGE₂, GM-CSF, and IL-8 release (Figure 2). However, although TNF-α stimulated increases in PGE₂ and IL-8 release by venous cells, no detectable increase in GM-CSF release was observed (Figure 2b). Excluding release of GM-CSF from arterial cells (Figure 1b), stimulation of cells with IL-1β increased PGE₂, GM-CSF, and IL-8 release to a greater extent than was observed with the same concentrations of TNF-α. Neither IFN-γ nor LPS had any effect on the release of PGE₂ or GM-CSF from arterial or venous smooth muscle cells (data not shown). However, LPS, but not IFN-γ, increased IL-8 release from both cell types (data not shown). Arterial cells released less PGE₂ and
more GM-CSF than did venous cells, basally and in the presence of inflammatory cytokines (Figures 1a, 1b, 2a, and 2b).

Effect of NSAIDs on PGE$_2$, GM-CSF, and IL-8 Release From Human Cultured Arterial and Venous Smooth Muscle Cells

Basal release of PGE$_2$ from arterial and venous cells was abolished by pretreatment (~5 minutes) with the COX inhibitor indomethacin (1.0×10$^{-5}$ mol/L; data not shown). In venous cells, basal release of GM-CSF was undetectable and remained undetectable in the presence of indomethacin (1.0×10$^{-5}$ mol/L). In arterial cells, basal release of GM-CSF was potentiated in the presence of indomethacin (8.6×$±$3.5 [n=36] versus 25.4×$±$9.7 [n=24] pg/mL). Indomethacin had no effect on basal release of IL-8 (data not shown).

Indomethacin (1.0×10$^{-7}$ to 1.0×10$^{-5}$ mol/L) caused a concentration-dependent decrease in PGE$_2$ together with a concentration-dependent increase in GM-CSF release in venous cells (Figure 3a and 3b, respectively) and arterial cells (for GM-CSF release, basal versus the maximum effect (E-max) seen 217.1×$±$83.9 versus 1132.5×$±$74.9 pg/mL; n=3) treated with IL-1$\beta$ (1 ng/mL). However, pretreatment of arterial and venous cells with indomethacin in the presence of IL-1$\beta$ or TNF-$\alpha$ had no effect on IL-8 release from either cell type (data not shown).

Pretreatment of venous cells with increasing concentrations of aspirin, nimesulide, meloxicam, and L-745,337 (Figure 4a, 4b, 4c, and 4d, respectively) in the presence of IL-1$\beta$ (1 ng/mL), as with indomethacin, produced a concentration-dependent inhibition of PGE$_2$ and potentiation of GM-CSF release. Similarly, treatment of arterial cells with increasing concentrations of these NSAIDs in the presence of IL-1$\beta$ (1 ng/mL) inhibited PGE$_2$ and potentiated GM-CSF release (for GM-CSF release, basal versus E-max 217.1×$±$83.9 versus 732.5×$±$71.4 pg/mL [aspirin], 217.1×$±$83.9 versus 709.6×$±$39.4 pg/mL [nimesulide], 217.1×$±$83.9 versus 807.4×$±$57.7 pg/mL [meloxicam], and 217.1×$±$83.9 versus 1263.3×$±$21.0 pg/mL [L-745,337]; n=3). In arterial or venous smooth muscle cells pretreated with indomethacin, nimesulide, meloxicam, and L-745,337, an inhibition of PGE$_2$ of >70% was required before a significant increase in GM-CSF release was observed. Furthermore, a complete inhibition of
PGE₂ release was required before GM-CSF release was maximal. However, in both cell types, pretreatment with aspirin resulted in GM-CSF release being maximum before PGE₂ release was maximally inhibited (Figure 4a).

**Effect of Cicaprost, PGE₂, and Dibutyryl cAMP on GM-CSF Release From Human Cultured Arterial and Venous Smooth Muscle Cells Stimulated With IL-1β and Indomethacin**

In venous cells (Figure 5) and arterial cells (data not shown), PGE₂ (1×10⁻⁸ to 1×10⁻⁵ mol/L), cicaprost (1×10⁻⁷ to 1×10⁻⁶ mol/L), and dibutyryl cAMP (1×10⁻⁸ to 1×10⁻⁴ mol/L) inhibited, in a concentration-dependent fashion, the increase in GM-CSF release observed from cells stimulated with IL-1β (1 ng/mL) in the presence of indomethacin (1×10⁻⁵ mol/L). It is worth noting that the endogenous levels of PGE₂ (9.1×10⁻⁵±2.8×10⁻⁵ mol/L [arterial] and 7.0×10⁻⁷±1.1×10⁻⁷ mol/L [venous]; see Figures 1a and 2a, respectively) released after 24 hours by both smooth muscle cell types stimulated with IL-1β were below those required exogenously (EC₅₀ >1.0×10⁻⁵ mol/L PGE₂, Figure 5) to inhibit GM-CSF release. In venous cells (Figure 5) and arterial cells (EC₅₀ 1.0×10⁻⁸ mol/L cicaprost), the PGI₃ mimetic cicaprost was more potent than PGE₂ at inhibiting GM-CSF release. This suggests that PGI₃, which is also released by these cells after stimulation by IL-1β, could be the endogenous COX-2 product that is limiting GM-CSF production.

**Discussion**

The “inflammatory response” is mediated by a number of distinct chemical messengers that achieve biological effects individually as well as in association with each other. PGE₂ and other COX metabolites have been implicated with inflammation for decades. Indeed, PGE₂ facilitates plasma exudation and pain. In many types of inflammatory response, there is a clear association with the presence of activated neutrophils, edema, and severity of tissue damage. Thus, the release of mediators that attract (eg, IL-8), activate, or increase the longevity (eg, GM-CSF) of neutrophils is likely to occur simultaneously with the release of PGE₂. The inflammatory mediators, PGE₂, GM-CSF, and IL-8, are thought to be primarily released by endothelial cells or leukocytes. However, in the present study, we show that human vascular smooth muscle cells release PGE₂, GM-CSF, and IL-8 spontaneously. In addition, we show that when stimulated with cytokines, human vascular smooth muscle cells release exaggerated amounts of these mediators. After stimulation with IL-1β, human venous smooth muscle cells released more PGE₂ than did equivalently treated arterial cells. This confirms our previous observations that related similar findings to a differential expression of COX-2 in the 2 cell types. By contrast, in the present study, we found that GM-CSF release was greater in arterial than venous cells. No appreciable difference was seen in the ability of arterial and venous cells to form IL-8.

The apparent inverse relation seen between the release of PGE₂ and GM-CSF by arterial and venous cells suggested to us that there might be interactions between these 2 mediators. Indeed, when COX activity was inhibited by indomethacin, there was a concomitant reduction in PGE₂ and increase in GM-CSF release. This observation is in keeping with the findings of others, who used a human lung fibroblast cell line stimulated with cytokines or human synovial fibroblasts stimulated with IL-1α. However, regarding human synovial
fibroblasts, it has been reported that indomethacin potentiated fibroblast IL-8 release; this finding contrasts with our own observations that the release of IL-8 from arterial and venous cells is unaffected in the presence of indomethacin. Our results suggest that in human cultured arterial and venous smooth muscle cells, COX products are involved in the mechanism of GM-CSF, but not IL-8, release. It has been reported that indomethacin has actions other than the inhibition of COX.19–21 Such reports introduce the possibility that our results are due to a direct action of indomethacin on GM-CSF synthesis. We have addressed this issue by repeating our experiments with a range of other NSAIDs substituted for indomethacin. In every case, the same concentration-dependent decrease in PGE2 (and corresponding increase in GM-CSF) release was observed, thus firmly establishing a role for COX in the mechanism of GM-CSF release.

The findings that COX activity differentially regulates GM-CSF versus IL-8 production suggest important differences in the regulation of these 2 genes. COX-2 is an immediate-early gene that is normally expressed transiently,22 whereas GM-CSF and IL-8 are thought to be continuously elevated in some inflammatory states.23 Thus, it is tempting to speculate that COX activity limits the survival-promoting GM-CSF until neutrophils are present, which are recruited by means of IL-8 production at the site of inflammation. Once present, COX activity may subside, resulting in a late burst of GM-CSF, which would be suitably timed for maximum impact in prolonging the survival of neutrophils.

Two isoforms of COX exist, COX-1 and COX-2.22 COX-1 is the constitutive form of the enzyme. COX-2, induced by inflammatory stimuli,24,25 is the predominant isoform present at sites of inflammation. In the present study, we find that in arterial and venous cells, the selective COX-2 inhibitor L-745,337,26 like all the NSAIDs tested, produces a concentration-dependent decrease in PGE2 release and an increase in GM-CSF release. This suggests that COX-2 products are modulating GM-CSF release in the present study. Our observations shed new light on previous studies showing that the COX-1/COX-2 inhibitor indomethacin increases GM-CSF release by human fibroblasts17,18 and suggest that in those studies, COX-2 was the isoform responsible for the apparent break in GM-CSF production.

In general, COX-1 and COX-2 perform the same enzymatic processes, forming PGG2 and PGH2. The profile of prostanooids produced by cells expressing either COX-1 or COX-2 is therefore dependent on the distribution of “downstream” synthase enzymes (eg, PGI2 synthase or thromboxane synthase) or the oxidative state of the cells. We have previously shown that when human venous or arterial cells are stimulated to express COX-2, they synthesize PGI2 and PGE2 predominantly, with very low levels of thromboxane.11 Thus, it is likely that PGI2 or PGE2 formed after COX-2 induction is suppressing GM-CSF release in human venous or arterial cells. Indeed, we found that either the PGI2 mimetic cicaprost27,28 or PGE2 was able to fully reverse the increase in GM-CSF release produced by indomethacin in cytokine-stimulated cells. The inhibitory action of PGE2 on GM-CSF mRNA and protein levels in human synovial fibroblasts,18 bone marrow stromal cells,29 and WI-38 lung fibroblasts17 has recently been reported. However, in human vascular cells, we found that cicaprost was ≈10 000-fold more potent than PGE2. Furthermore, the endogenous levels of PGE2 released by both by smooth muscle cell types stimulated with IL-1β were below those required exogenously to inhibit GM-CSF release, suggesting the involvement of PGI2 receptors in this response.

Many of the biological effects of PGI2 are mediated by the second messenger, cAMP.30,31 Indeed, PGI2 receptors are linked to adenylyl cyclase, the activation of which leads to elevated levels of cAMP. In the present study, we observed that the cell-permeable cAMP analogue, dibutyryl cAMP, similar to cicaprost, inhibited the effects of indomethacin on GM-CSF release from cells stimulated with IL-1β. These observations are in keeping with others recently published showing that dibutyryl cAMP inhibits GM-CSF expression in human bone marrow stromal cells29 and lung fibroblasts.17

PGI2 is a vasodilator with potent inhibitory actions on platelet function.32–34 Infusions of PGI2 or PGI2 mimetics are used therapeutically in the management of primary pulmonary hypertension and peripheral vascular disease and as an alternative to heparin infusion in hemofiltration. It has also been reported that PGI2 has antilipidemic properties35 and antimitotic actions on vascular smooth muscle cells36 and, thus, may be useful in the prevention/treatment of atherosclerosis. HDLs, the low plasma levels of which are indicative of atherogenesis, have been shown to increase COX-2 expression, inducing PGI2, in cytokine-stimulated endothelial cells.37 In many vascular diseases, including atherosclerosis, damage to the vessel wall leads to the recruitment and activation of inflammatory cells. Neutrophils are the first inflammatory cells to appear at the site of vessel damage, exacerbating and propagating the inflammatory response. It has also been reported that neutrophils generate reactive oxygen species, which may cause lipoprotein lipid oxidation, thereby contributing to the pathogenesis of atherosclerosis.38–40 Thus, we suggest that some of the anti-inflammatory/cytotoxic actions of PGI2 occur by inhibiting neutrophil survival as a direct consequence of reducing GM-CSF release at the site of inflammation.

In conclusion, we have shown that human vascular smooth muscle cells are a rich source of IL-8, GM-CSF, and COX-2 products. Moreover, we have identified and characterized an inhibitory pathway by which COX-2 activity suppresses GM-CSF (but not IL-8) release. Because GM-CSF promotes activation, surface receptor expression, and the survival of circulating mature neutrophils,3 these observations may help to explain some of the side effects associated with NSAIDs that are in current use. Moreover, we suggest that COX-2 inhibitors will also increase GM-CSF release under certain circumstances, an effect that may lead to side effects with these drugs. Our findings that cicaprost potently inhibits GM-CSF release may help to explain the mechanism of cytotoxicity associated with PGI2 vascular diseases, such as pulmonary hypertension and peripheral vascular disease, in which neutrophil activation contributes to the disease.

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