Negative Feedback Between Prostaglandin and α- and β-Chemokine Synthesis in Human Microglial Cells and Astrocytes

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Negative Feedback Between Prostaglandin and \(\alpha\) -  and \(\beta\)-Chemokine Synthesis in Human Microglial Cells and Astrocytes

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The understanding of immune surveillance and inflammation regulation in cerebral tissue is essential in the therapy of neuro-immunological disorders. We demonstrate here that primary human glial cells were able to produce \(\alpha\)- and \(\beta\)-chemokines (IL-8 > growth related protein \(\alpha\) (GRO\(\alpha\)) > RANTES > microphage inflammatory protein (MIP)-1\(\alpha\) and MIP-1\(\beta\)) in parallel to PGs (PGE\(\alpha\) and PGF\(\alpha\)) after proinflammatory cytokine stimulation: TNF-\(\alpha\) + IL-1\(\beta\) induced all except RANTES, which was induced by TNF-\(\alpha\) + IFN-\(\gamma\). Purified cultures of astrocytes and microglia were also induced by the same combination of cytokines, to produce all these mediators except MIP-1\(\alpha\) and MIP-1\(\beta\), which were produced predominantly by astrocytes. The inhibition of PG production by indomethacin led to a 37–60% increase in RANTES, MIP-1\(\alpha\), and MIP-1\(\beta\) but not in GRO\(\alpha\) and IL-8 secretion. In contrast, inhibition of IL-8 and GRO activities using neutralizing Abs resulted in a specific 6-fold increase in PGE\(\alpha\) but not in PGF\(\alpha\) production stimulated microglial cells and astrocytes, whereas Abs to \(\beta\)-chemokines had no effect. Thus, the production of PGs in human glial cells down-regulates their \(\beta\)-chemokine secretion, whereas \(\alpha\)-chemokine production in these cells controls PG secretion level. These data suggest that under inflammatory conditions, the intraparenchymal production of PGs could control chemotactic gradient of \(\beta\)-chemokines for an appropriate effector cell recruitment or activation. Conversely, the elevated intracerebral \(\alpha\)-chemokine levels could reduce PG secretion, preventing the exacerbation of inflammation and neurotoxicity. The Journal of Immunology, 1999, 162: 1701–1706.

Inflammatory processes resulting from an intricate network of mediators play a central role in several central nervous system (CNS)\(^4\) diseases. The proinflammatory cytokines induce astrocytes and microglial cells to secrete several inflammatory mediators such as chemokines, lipid mediators, and free radicals (1–6). Chemokines are involved in the activation and recruitment of monocytes and lymphocytes into the brain parenchyma (7). Products of arachidonic acid metabolism include prostanooids (derived from the cyclooxygenase pathway) that are involved in the control of body temperature, neuroendocrine function, sleep, cerebral ischemia, brain edema, and inflammation (8–12). Prostanoids are also implicated in chemotaxis as well as in the modulation of blood flow and vascular permeability (12, 13). Although these inflammatory mediators are all constitutively expressed in brain parenchyma for regulation of normal CNS activities, their production has to be under strict control to prevent inflammatory tissue damage. Thus, cascade production of cytokines, positive and negative feedback, and synergistic mechanisms are parameters that characterize the action of these mediators in the inflammatory process.

The role of chemokines and PGs in several biological effects induced by proinflammatory cytokines, as well as the presence of common events in the pathways of synthesis and signal transduction of these two classes of mediators such as phospholipase A2 activation and intracellular calcium mobilization (14, 15), prompted us to study a possible interaction between chemokine and PG pathways of synthesis. In this report, we demonstrate that human microglia and astrocytes produce \(\alpha\)- and \(\beta\)-chemokines (IL-8, growth related protein\(\alpha\) (GRO\(\alpha\)), RANTES, microphage inflammatory protein (MIP)-1\(\alpha\), and MIP-1\(\beta\)) and PGs under proinflammatory conditions. The production of \(\beta\)-chemokines in these cells is negatively regulated by their PG secretion, whereas PG production itself is limited by \(\alpha\)-chemokine secretion.

Materials and Methods

Primary cultures of human CNS cells

Eight- to 10-wk-old human embryos were obtained after elective abortion in compliance with the recommendations of the French National Ethics Committee and following approval by the local ethics committee. Primary cultures of spinal cord and prosencephalon were prepared as described (16, 17). Briefly, tissues were dissected, trypsinized, and resuspended in MCDB 153 medium (Polylabo, Paris, France) completed by 5% FCS, 2 mM glutamine, 10\(^5\) U/liter penicillin, and 0.1 g/liter streptomycin. The cell suspension was seeded at a density of 5 × 10\(^5\) cells per well (24-well plates, Nunc, Roskilde, Denmark) coated with collagen (100 \(\mu\)g/ml). Cultures were kept at 37°C in a 10% CO\(_2\) atmosphere. The medium was changed completely after 48 h and then by half every 3 days. These cultures consisted of neurofilament-positive neuronal clusters lying on a monolayer of astrocytes and 40% CD68-positive microglial cells associated with <10% fibroblast-positive fibroblasts (16–18).

Purified cultures of human microglial cells and astrocytes

To obtain purified microglial and astrocyte cultures, CNS cells were seeded at a density of 2 × 10\(^5\) cells per well in 6-well plates. After plating (10–15 days), the nonadherent fraction was washed with minimal essential medium (MEM) supplemented with 0.5% fetal calf serum (FCS) and 10% serum-free FCS (SF-FCS) and harvested by trypsinization.
days), the microglial cells were released by circular shaking, followed by a 20-min incubation at 37°C to select adherent cells, as described (17). These cells were subsequently grown in the same medium as the primary mixed cultures for 3 wk to 2 mo to obtain a monolayer containing >95% CD68/KiM-7 and EBM11-positive cells (17). The adherent cells remaining after release of microglial cells were trypsinized, plated, and passaged two to four times to obtain purified cultures of astrocytes (>95% GFAP-positive cells) (17). Under the usual experimental conditions, 5 × 10^5 astrocytes or microglial cells were seeded in 24-well plates and stimulations were performed at confluence.

Cell stimulations and reagents
To stimulate cultures, cells were refed with serum-free MCDB 153 medium containing the tested inducers, inhibitors, or Abs. Abs. IL-1β (200 U/ml) was purchased from Genzyme (Cambridge, MA), TNF-α (200 U/ml) and IFN-γ (200 U/ml) were purchased from Boehringer Mannheim (Maylan, France). All are recombinant human cytokines. Human rIL-8, GROα (500 ng/ml), and neutralizing Abs against human RANTES (polyclonal, 200 μg/ml), MIP-1α and MIP-1β (polyclonal, 100 μg/ml) (19–22), IL-8, and GRO (monoclonal, 100 μg/ml) were purchased from R&D Systems (Minneapolis, MN). The specific inhibitor for cyclooxygenase (indomethacin, 5–15 μM) was purchased from Sigma (St. Louis, MO). PGF2α and PGE2 were purchased from Cayman Chemicals (Ann Arbor, MI, PGF2α, and PGE2, 16 ng/ml) (23).

Chemokine and PG titrations
The concentrations of all tested mediators were measured by enzyme immunoassay (PGF2α and PGE2, Cayman Chemicals; α- and β-chemokines, R&D Systems). All assays were performed using standards and instructions supplied by the manufacturers.

Statistical analysis
Each measurement was performed at least in triplicate. Student’s t test was used for determination of statistical significance of two to five independent experiments. Results were considered significant at p ≤ 0.05.

Results
Induction of α- and β-chemokine production by proinflammatory cytokines in human glial cultures
Spontaneous IL-8 and GROα production was detected in the supernatant of mixed glial cultures. The effect of proinflammatory cytokines on this production was tested in the absence of serum: IL-1β and, to a lesser extent, TNF-α, but not IFN-γ alone led to an increase in IL-8 and GROα production by glial cells at day 3 poststimulation (Fig. 1A). However, a combination of TNF-α + IL-1β was most efficient at inducing α-chemokine production (IL-8: 290,435 ± 33,483 vs 2, 704 ± 1,590 pg/ml; GROα: 64, 978 ± 10, 884 vs 1, 516 ± 977 pg/ml, (TNF-α + IL-1β)-stimulated vs control, n = 5, p < 0.0001, Fig. 1A). RANTES secretion by mixed glial cultures was also induced in the presence of proinflammatory cytokines although the induction profile of the cytokine combinations differed (IFN-γ + TNF-α > IFN-γ + IL-1β > TNF-α + IL-1β, Fig. 1B). IL-1β, TNF-α, or IFN-γ alone had no significant effect on RANTES production by these cells. The level of RANTES production by glial cells was at least 10-fold lower than that of α-chemokines (4,958 ± 1, 190 vs 7 ± 0.3 pg/ml, (IFN-γ + TNF-α)-stimulated vs control, day 3, n = 3, p = 0.001). The same cytokines induced production of MIP-1α and MIP-1β in mixed glial cultures (TNF-α + IL-1β > IFN-γ + IL-1β = IFN-γ + TNF-α, Fig. 1C), which nonetheless remained low compared with RANTES (MIP-1α: 881 ± 88 vs 11 ± 1 pg/ml; MIP-1β: 1, 345 ± 159 vs 36 ± 7 pg/ml, (TNF-α + IL-1β)-stimulated vs control, day 3, n = 3, p < 0.0001). Kinetics of IL-8, GROα, RANTES, MIP-1α, and MIP-1β production by cytokine-stimulated mixed glial cells demonstrated a similar profile of chemokine induction starting between 6 and 24 h poststimulation and accumulating over the 3 days tested (Fig. 2). All purified cultures of both microglia and astrocytes tested secreted high levels of IL-8, GROα, and RANTES, respectively, after TNF-α + IL-1β and IFN-γ + TNF-α stimulation (Fig. 3, A and B). In contrast, only two of six purified cultures of microglia could be induced by TNF-α + IL-1β to produce MIP-1α, and three of six produced MIP-1β, whereas four of five purified cultures of astrocytes were induced to secrete both MIP-1α and MIP-1β under the same condition of stimulation (Fig. 3C). Accordingly, MIP-1α and MIP-1β secretion was induced more efficiently in purified cultures of astrocytes compared with microglia. Nevertheless, the level of MIP-1α and MIP-1β secretion in purified cultures of astrocytes remained low in comparison with mixed glial cultures.

Regulation of β-chemokine production by PGs and of PG production by α-chemokines in human glial cells
To investigate the possible involvement of PGs in the regulation of chemokine production by glial cells, we first tested the induction of PGF2α and PGE2 in the supernatant of mixed glial cultures after proinflammatory cytokine stimulation (PGE2: 19, 814 ± 4, 315 vs 421 ± 230 pg/ml; PGF2α: 23,596 ± 3,739 vs 735 ± 268 pg/ml, (TNF-α + IL-1β)-stimulated vs control, day 3, n = 5, p < 0.0001, Fig. 1D). The induction of PG production was inhibited in the
FIGURE 2. Kinetics of α- and β-chemokine production in human mixed glial cultures during the 3 days poststimulation with proinflammatory cytokines (○, control; ●, TNF-α + IL-1β; ▲, IFN-γ + TNF-α). The increase in GROα, IL-8, MIP-1α, MIP-1β, and RANTES production started between 6–8 h after stimulation of glial cells with a combination of TNF-α + IL-1β and IFN-γ + TNF-α, respectively, and accumulated until day 3 post-stimulation. Results are the mean ± SD of two independent experiments using cells from two different brain specimens.

presence of 5 μM indomethacin, a specific cyclooxygenase inhibitor (PGE2: 12,773 ± 2,098 vs 147 ± 24 pg/ml; PGF2α: 13,983 ± 2,144 vs 287 ± 25 pg/ml, (TNF-α + IL-1β)-stimulated vs (TNF-α + IL-1β + indomethacin)-treated). Both microglial cells and astrocytes were responsible for PG production as demonstrated previously (data not shown; see also Ref. 17). The effect of indomethacin was subsequently tested on the induced production of chemokines. Treatment of mixed glial cultures with 5–15 μM indomethacin at the time of stimulation led to a 37–60% increase in the production of RANTES, MIP-1α, and MIP-1β induced, respectively, by IFN-γ + TNF-α and TNF-α + IL-1β (Fig. 4, A and B; RANTES: 4,142 ± 597 vs 6, 100 ± 674; MIP-1α: 420 ± 56 vs 661 ± 57; MIP-1β: 602 ± 45 vs 894 ± 52 pg/ml, cytokine-treated vs (cytokine + 5 μM indomethacin)-treated, day 3, n ≥ 4, p ≤ 0.03). The effect of indomethacin was dose-dependent in the range of the tested concentrations. This suggested a limitation of β-chemokine secretion by human glial cells in the presence of PGs. Addition of exogenous PGF2α, and PGE2 to the culture supernatants abolished the effect of indomethacin on this chemokine production (Fig. 4, A and B), indicating a direct effect of PGs. In contrast, inhibition of PG secretion by indomethacin had no effect on GROα and IL-8 production in (TNF-α + IL-1β)-stimulated glial cells (Fig. 4C). To investigate the role of microglia and astrocytes in the regulation of β-chemokines by PGs, purified cultures of microglial cells and astrocytes were analyzed under the same conditions. Although all purified microglial or astrocyte cultures were induced with IFN-γ + TNF-α to produce RANTES, this production was not significantly modified after inhibition of PG secretion by indomethacin (not shown). In contrast, indomethacin treatment of astrocytes led to a significant increase in MIP-1α and MIP-1β production by these cells after TNF-α + IL-1β stimulation (Fig. 5).

To determine whether α- or β-chemokines could also regulate PG secretion by glial cells, we tested the effect of neutralization of chemokine activities on the induced production of PGs. Neutralization of GROα and/or IL-8, but not of β-chemokines, in the presence of neutralizing Abs led to a strong increase in the production of PGE2 by mixed glial cultures (Fig. 6A, 23,969 ± 7,500 vs 147,218 ± 14,320 pg/ml, cytokine-treated vs (cytokines + anti-IL-8 + anti-GRO)-treated, day 3, n = 4, p < 0.0001), whereas PGF2α secretion remained unchanged (Fig. 6). The enhancement of PGE2 levels in mixed glial cultures, in the presence of anti-IL-8 and anti-GRO Abs, was due to an increase in PGF2α production in both microglia and astrocytes (Fig. 6). The treatment of unstimulated glial cells by purified IL-8 and GROα did not significantly affect their spontaneous production of PGF2α or PGE2α (data not shown).

Discussion

The understanding of the different interactions between inflammatory mediators leading to a restricted local inflammatory reaction is important in the development of novel therapies to treat inflammatory CNS diseases. Our results describe the parallel production of α- and β-chemokines in human microglial cells and astrocytes. This is also the first demonstration of a negative retroregulatory loop between α- and β-chemokine and PG production in human glial cells.

Studies on chemokine production by CNS resident cells of either human or rodent origin have focused mainly on a single class of chemokine in either microglial cells or astrocytes (5, 6, 24–28). Here we describe the production of high amounts of IL-8 and GROα by microglia and astrocytes, either in close interaction or isolated, after stimulation with TNF-α in combination with IL-1β. Mixed glial cells were also induced by the same cytokines to secrete MIP-1α and MIP-1β but to a lesser extent compared with α-chemokines. This production was probably due to astrocytes rather than microglia because 80% of the astrocyte cultures tested could be induced to secrete MIP-1α and MIP-1β compared with 20–30% of microglial cultures. However, the observation of a higher level of MIP-1 production in mixed glial cultures than in astrocytes suggests that cell interactions between microglia and astrocytes facilitate MIP-1α and MIP-1β production. The difference in the ability of astrocytes and microglia to secrete MIP-1α and MIP-1β is reminiscent of previous data describing the inability of rodent microglial cells to produce monocyte chemotactic protein-1 (MCP-1) protein, whereas astrocytes from the same origin secreted MCP-1 under the same condition of stimulation (25). Moreover, it has been shown that LPS-stimulated murine alveolar and peritoneal macrophages expressed MIP-1α mRNA, whereas
only alveolar macrophages could produce MIP-1α protein in their supernatant after stimulation (29). The disparity in MIP-1α secretion between these two populations of macrophages was attributed to an impairment in MIP-1α protein translation and secretion in peritoneal macrophages (29). Another explanation for the differentially expressed MIP-1α and MIP-1β by astrocytes and microglia could be a difference in transcription factors between the two cell types such as activating transcription factor/cAMP response element binding protein (ATF/CREB)-, activating protein-1 (AP-1)-, NF-κB-, CCAAT/enhancer binding protein (C/EBP), cellular-E26-specific (C-ET)-related proteins, and/or MIP-1α nuclear protein (30–32). However, the possibility remains that other stimuli are required for an efficient induction of these β-chemokines in microglial cells. Finally, the production of RANTES, which was less than that of α-chemokines but more than that of MIP-1α and MIP-1β, was increased in mixed or isolated glial cultures after stimulation by TNF-α in combination with IFN-γ rather than IL-1β. The fact that the induction of different chemokines did not require the same combination of cytokines suggests different mechanisms of induction. The inducing effect of inflammatory cytokines might involve the induction of different transcription factors such as STAT1, NF-κB, or AP-1, as shown in certain cell types (33–36).

**FIGURE 3.** α- and β-chemokine production in purified cultures of human microglial cells and astrocytes. Both microglial cells and astrocytes were able to produce GROα, IL-8 (A) MIP-1α, MIP-1β (C), and RANTES (B) at day 3 poststimulation with TNF-α + IL-1β and IFN-γ + TNF-α, respectively. Five independent experiments were performed in triplicate. Results shown are the mean ± SD of five independently isolated glial cultures (for RANTES), of three and two of six isolated microglial cultures (for MIP-1α and MIP-1β, respectively) and of four out of five isolated astrocyte cultures (for MIP-1α and MIP-1β).

**FIGURE 4.** Effect of PG secretion blockade by indomethacin on proinflammatory cytokine-induced production of α- and β-chemokines in human mixed glial cultures. A, IFN-γ + TNF-α-induced RANTES production was increased by 37% in the presence of 15 μM indomethacin. This increase was reversed after exogenous addition of PGE2α and PGE2 (16 ng/ml). B, The 47% increase in (TNF-α + IL-1β)-induced MIP-1α and MIP-1β production in the presence of 15 μM indomethacin was also reversed after addition of PGE2α and PGE2. C, Inhibition of PG production in (TNF-α + IL-1β)-stimulated glial cells had no effect on their GROα and IL-8 production. Mean ± SD of three to five independent experiments performed in triplicate, using cells from four to five different brain specimens.

**FIGURE 5.** Effect of PG secretion blockade by indomethacin on proinflammatory cytokine-induced production of MIP-1 in purified cultures of human astrocytes. MIP-1α- and MIP-1β-induced production was increased by $40\%$ in the presence of indomethacin. Mean ± SD of four independent experiments using four different isolated astrocyte cultures.
Numerous in vitro studies have demonstrated the role of $\alpha$- and $\beta$-chemokines in inducing the migration of specific populations of leukocytes (6, 25, 27, 37). However, in vivo studies have demonstrated that the physiological functions of these chemokines are highly dependent on their level of production. In fact, transgenic mice overexpressing IL-8 exhibited a marked circulating neutrophilia and a decreased neutrophilic exudation into body cavities in response to acute inflammatory stimulants (38). Similarly, transgenic mice overexpressing MCP-1 displayed no monocyte infiltration into tissues and showed increased sensitivity to intracellular pathogens (39). In contrast, mice in which murine GRO $\alpha$ or MCP-1 were expressed at lower levels and in anatomically restricted areas displayed an appropriate infiltration of neutrophils or monocytes to the site of chemokine production (40, 41). Thus, overproduction of chemokines in vivo might lead to a loss of their specific biological effect through either the inactivation of leukocytes by receptor desensitization or the abolition of the chemotactic gradient.

We investigated whether chemokine production could be under the control of other inflammatory mediators such as PGs, secreted in parallel in proinflammatory cytokine-stimulated glial cells. Inhibition of PG production in glial cells by the cyclooxygenase inhibitor, indomethacin, resulted in an increase in glial secretion of PGE$_2$ but not of PGF$_2\alpha$. The addition of Abs to neutralize $\alpha$-chemokines, leading to an appropriate activation of monocytes and lymphocytes, and/or an appropriate recruitment of these cells to the inflammatory response (Fig. 6).

The immune surveillance in CNS inflammation has to be tightly controlled so as to mount an efficient defense against pathogens. An imbalance between immunoregulatory factors during infectious or autoimmune diseases may have drastic effects on the integrity of the brain parenchyma. In this respect, studies of anti-inflammatory treatments in different in vivo experimental systems are of great interest. Although in such models, anti-inflammatory drugs generally lead to the inhibition of vasoactive product secretion by effector cells (42–44), the inconsistency of their effect on the neurological lesions reflects subtle and time-dependent retroregulations, which determine the final biological effect on neuron function and survival.

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References


