Induction of macrophagic prostaglandin E₂ synthesis by glioma cells

YOSHITERU NAKANO, M.D., ETSUBI KURODA, PH.D., TOMOHIRO KITO, M.D., PH.D., AKIRA YOKOTA, M.D., PH.D., AND UKI YAMASHITA, M.D., PH.D.

Departments of Neurosurgery and Immunology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu; and Department of Neurosurgery, Niigata Rosai Hospital, Jyoetsu, Japan

Object. It has been reported that glioma cells produce prostaglandin (PG)E₂, which promotes the growth of tumor cells and possesses immunosuppressive activity, and that cyclooxygenase (COX) inhibitors impede tumor growth and infiltration. Macrophages in tumor-bearing hosts are activated to produce PGE₂, which induces an immunosuppressive state. However, the precise mechanism by which PGE₂ induces an immunosuppressive state is still unclear. In this study, the authors investigated the mechanism of PGE₂ production in glioma-bearing hosts.

Methods. The human and murine glioma cells that were studied did not produce a significant amount of PGE₂. However, the coculture of human peripheral blood mononuclear cells or murine peritoneal macrophages with glioma cells or conditioned glioma medium led to the production of a large amount of PGE₂. In contrast, production of tumor necrosis factor and interleukin (IL)-12p70 by macrophages and cytotoxic T lymphocyte induction were suppressed by cultivating with conditioned glioma medium; this suppression was abrogated by the addition of the COX inhibitor indomethacin. The macrophagic expression of COX-2, and particularly the expression of microsomal PGE synthase (mPGES)-1, a terminal enzyme of the arachidonate cascade, was enhanced by the glioma-derived soluble factors. Furthermore, IL-12p70 production was not clearly suppressed in macrophages from mPGES-1–deficient mice. The glioma-derived soluble factors were sensitive to treatment with heat and papain.

Conclusions. These results indicated that PGE₂ production by macrophages is enhanced by glioma-derived soluble factors, which induce an immunosuppressive state in glioma-bearing hosts. Therefore, the inhibition of PGE₂ synthesis, targeting COX-2 and mPGES-1, is an effective treatment for the induction of anti-glioma immune responses.

Key Words • glioma • macrophage • prostaglandin • tumor immunity • mouse

A mong tumors, malignant glioma causes one of the most severe diseases. Although extensive treatments, including resection, irradiation, chemotherapy, and immunotherapy, have been tried, the prognosis is very poor. It is known that various immunogenic cells, such as T, B, and natural killer cells and macrophages, are activated in glioma-bearing hosts. It is difficult to overcome malignant glioma with immune responses alone, however, given that an immunosuppressive state is induced in the glioma-bearing host. This immunosuppressive state is induced by tumor-derived soluble factors such as PGE₂ or TGFβ. It has been reported that COX-2 is overexpressed in glioma cells and that COX-2 inhibitors express an antitumor effect against various tumor cells. In contrast, it has been reported that the activated macrophages around tumor tissues also exert suppressive effects on immune responses by producing PGE₂; these macrophages are called “suppressor macrophages.” The mechanism of induction and activity of suppressor macrophages in tumor-bearing hosts are still not clear, however.

Prostaglandin E₂ is a small lipid molecule that has regulatory roles in the immune system. It is produced by various kinds of cells, including tumor cells. In the immune system, PGE₂ is mainly produced by antigen presenting cells, such as monocytes, macrophages, and dendritic cells, and the main effects of PGE₂ are the suppression of T helper 1–related immune responses and the augmentation of T helper 2–related immune responses.

Prostaglandin E₂ is synthesized in three sequential steps. First, arachidonic acid is liberated from membrane phospholipids by the action of cPLA₂ or sPLA₂ isoenzymes. Next, released arachidonic acid is converted to PGH₂ by two COX isozymes, COX-1 and COX-2. Finally, PGH₂ is converted to PGE₂ by cPGES, mPGES-1, or mPGES-2. Microsomal prostaglandin E synthase–1 is a member of the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily. It has been suggested that the aberrant expression of mPGES-1 in combination with COX-2 can contribute to tumorigenesis. In
Prostaglandin E₂ production by glioma-treated macrophages

In this study, we investigated the mechanism of PGE₂ production and the induction of suppressor macrophages in the glioma-bearing state.

Materials and Methods

Study Population

Seven- to 10-week-old female C57BL/6N and C3H/HeN mice were purchased from a commercial provider (Seac, Ohiita, Japan). The mPGES-1-deficient female C57BL/6 mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan).41 These mice were maintained in the Animal Research Center at the University of Occupational and Environmental Health, Kitakyushu, Japan, under specific pathogen-free conditions. All animal experiments were performed according to the guidelines for the care and use of animals approved by the University of Occupational and Environmental Health.

Reagents and Cell Lines

Indomethacin, anti-COX-2 antibody, anti–mPGES-1 antibody, and anti–cPGES antibody were purchased from Cayman Chemicals (Ann Arbor, MI). The LPS (Escherichia coli serotype 055:B5) and papain were purchased from Sigma-Aldrich (St. Louis, MO).

We used U251, human malignant astrocytoma, T98G, human glioblastoma multiforme; VM glioma; spontaneously induced astrocytoma originating in VM mice (H-2²); RSV-M: malignant glioma originating in C57/HeN mice (H-2²) and induced by Schmitt-Ruppin RSV; and X5653, plasmacytoma originating in C57/HeN mice (H-2²). These cell lines were continuously maintained in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FCS (BioWhittaker, Walkersville, MD), 2 mM glutamine, and 50 μg/ml streptomycin (all from Life Technologies, Rockville, MD). The VM glioma was provided by Dr. T. Yamasaki (Shimane University, Shimane, Japan),28 and the RSV-M was provided by Dr. K. Shimizu (Kochi University, Kochi, Japan).45

Preparation of Human PBMCs

Human PBMCs were isolated from healthy donors by using density gradient centrifugation with lymphocyte separation medium (Nakarai Tesque, Kyoto, Japan). Cells were collected at the interface, washed twice with Hank's balanced salt solution (Nissui Pharmaceutical), and resuspended in RPMI–10% FCS medium. Human PBMCs (2 × 10⁵/500 μl/well), human glioma cells (5 × 10⁵/500 μl/well), and mixtures of these cells were cultured in a 24-well culture plate for 6 hours, and then treated with or without LPS (0.1 μg/ml) for an additional 18 hours at 37°C in 5% CO₂ and 95% air. The cell-free culture supernatants were collected and used for the PGE₂ assay, as described later.

Preparation of Macrophages

Peritoneal exudate cells were harvested from C57BL/6N or mPGES-1-deficient C57BL/6 mice 3 days after the intraperitoneal injection of 2 ml 4% thioglycolate broth (Eiken, Tokyo, Japan). Cells were resuspended in RPMI–10% FCS medium, 2 mM glutamine, 50 μg/ml streptomycin, and 50 μg/ml penicillin and were seeded into 24-well plates (Falcon 3047; BD Biosciences, Franklin Lakes, NJ) or six-well plates (Falcon 3046; BD Biosciences). The cells were then cultured at 37°C in 5% CO₂ for 1.5 hours, nonadherent cells were washed out with warm Hank's balanced salt solution, and adherent cells were used for macrophage cultures. This adherent cell population contained more than 95% Mac-1⁺ cells, as detected by flow cytometry.

Preparation of Conditioned Glioma Medium

The VM glioma and RSV-M (2 × 10⁶/ml) were cultured in RPMI-1640 medium with or without 10% FCS at 37°C in 5% CO₂ and 95% air for 48 hours, and the supernatant was used as the conditioned glioma medium after filtration through a 0.2-μm cellulose acetate membrane (Millipore, Tokyo, Japan).
sentative result from each experiment is shown in the figures. All determinations were made in triplicate and each result was expressed as the mean ± standard error of the mean. Statistical analyses were performed using the Student t-test, and a confidence level of less than 0.05 was considered significant.

Results
Enhancement of PGE₂ Production by Murine Macrophages Cocultured With Glioma

At first, we investigated PGE₂ production by murine macrophages cocultured with glioma cells. Peritoneal macrophages were cocultured with glioma cells treated with or without LPS. As shown in Fig. 1, glioma cells produced only a small amount of PGE₂ by themselves. The PGE₂ production by macrophages was markedly enhanced by coculturing with glioma cells. Stimulation with LPS further enhanced PGE₂ production. This enhancement of PGE₂ production by macrophages depended on the number of glioma cells.

Soluble Mediators From Glioma Enhanced Macrophagic PGE₂ Production

To study the mechanism of the enhancement of PGE₂ production by macrophages cocultured with glioma cells, we performed experiments using a cell culture insert. Macrophages were cocultured with glioma cells treated with or without LPS in the presence or absence of a cell culture insert. The PGE₂ production by macrophages was significantly enhanced by coculturing with glioma cells regardless of whether a cell culture insert was present (Fig. 2A and B), suggesting that direct contact between macrophages and glioma cells is not required to enhance PGE₂ production. To confirm this phenomenon, we used conditioned glioma medium. As shown in Fig. 2C and D, PGE₂ production by macrophages was dose-dependently enhanced by the conditioned glioma medium. These results suggest that the enhancement of PGE₂ production by macrophages is mediated by soluble factors in the conditioned glioma medium.

Western Blot Analysis

Cells were lysed with 100 μl sodium dodecyl sulfate (Nacarai Tesque). The lysates were boiled for 10 minutes, resolved by 20% SDS-PAGE (Nacarai Tesque) with e-PAGE (ATTO, Tokyo, Japan), transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories), and blotted with anti–COX-2 antibody (1000 × dilution), anti–PGES-1 antibody (1000 × dilution), or anti–cPGES (1000 × dilution), and horseradish peroxidase–conjugated secondary antibody (1000 × dilution). The reactive bands were visualized using Lumi-LightPLUS Western blotting substrate (Roche Diagnostics, Indianapolis, IN) as a substrate and Fluorochem (Alpha Innotech, San Leandro, CA) as a detector.

Statistical Analysis

All experiments were repeated at least three times, and one representative result from each experiment is shown in the figures. All determinations were made in triplicate and each result was expressed as the mean ± standard error of the mean. Statistical analyses were performed using the Student t-test, and a confidence level of less than 0.05 was considered significant.
Prostaglandin E₂ production by glioma-treated macrophages

Fig. 2. Bar graphs demonstrating enhanced PGE₂ production by macrophages treated with soluble mediators from glioma. Macrophages (5 x 10⁵) were cocultured with glioma cells (5 x 10⁴) for 6 hours, and then stimulated with (black bars) or without (white bars) LPS (1 µg/ml) in the presence or absence of cell culture insert for 18 hours (A and B), or were cocultured with conditioned glioma medium (50, 25, and 10%) for 6 hours, and then stimulated with (black bars) or without (white bars) LPS (1 µg/ml) for 18 hours (C and D). Production of PGE₂ was detected using EIA. Production was significantly enhanced compared with the control group (*p < 0.01). RSVsup. = RSV supernatant; VMsup. = VM supernatant.

Fig. 3. Bar graphs depicting the characteristics of glioma-derived soluble factors. Conditioned glioma medium was treated at 56 C or 100 C for 30 minutes, and PGE₂ production by macrophages cultured with the heat-treated conditioned medium plus LPS (1 µg/ml) was compared with production induced by the untreated conditioned medium plus LPS (A and B). Conditioned glioma medium was treated (black bars) or not treated (white bars) with papain (0.2 mg/ml) for 24 hours. The PGE₂ production by macrophages cultured with the papain-treated medium plus LPS (1 µg/ml) was compared with the production induced by the untreated conditioned medium plus LPS (C). Production was significantly reduced compared with the untreated group (*p < 0.01).

Important cytokines for anti-tumor effects, is suppressed by enhanced macrophagic PGE₂ production.

**Suppression of Cytotoxic T Cell Induction by the Enhanced PGE₂ Production**

The TNP-specific cytotoxic T cells were induced by the culture of responder spleen cells with TNP-modified stimulator peritoneal macrophages in the presence or absence of conditioned glioma medium. Prostaglandin E₂ production was again enhanced by the presence of conditioned glioma medium (Fig. 5A and C). The TNP-specific cytotoxic T cells were suppressed by the presence of the conditioned medium; this suppression was overcome by indomethacin (Fig. 5B and D). These results indicated that the induction of cytotoxic T cells is suppressed by the enhanced production of PGE₂ by macrophages treated with the conditioned glioma medium.

**Expression of COX-2 and mPGES-1 in Macrophages Treated With Conditioned Glioma Medium**

It is known that COX-2 and mPGES-1 are inducible enzymes and that mPGES-1 is preferentially coupled with inducible COX-2 to promote the final step in PGE₂ biosynthesis. We examined whether the expressions of COX-2, mPGES-1, and other PGE₂ biosynthetic enzymes were induced by the conditioned glioma medium. The expression of both COX-2 and mPGES-1 was augmented by the conditioned glioma medium.
ditioned glioma medium, with the expression of mPGES-1 being more markedly enhanced. Note, however, that the expression of cPLA₂, sPLA₂ Type V, COX-1, and cPGES/p23 was not changed (Fig. 6A and B).

Prostaglandin E₂ and IL-12 Production in Macrophages From mPGES-1–Deficient Mice

To confirm the role of mPGES-1 in the suppression of the antiglioma effect, we studied PGE₂ and IL-12 production by macrophages treated with conditioned glioma medium. Macrophages (5 × 10⁵) were cocultured with conditioned glioma medium in the presence or absence of indomethacin (1 μM) for 6 hours and then treated with (black bars) or without (white bars) LPS (1 μg/ml) for 18 hours. Production of TNF was detected using an L929 bioassay (A and B). Production of IL-12 was detected using ELISA (C and D). Production of PGE₂ was detected using EIA (E and F). Production was significantly suppressed compared with the control group (*p < 0.01). Production was significantly enhanced compared with the indomethacin group (†p < 0.01 and ††p < 0.05). Indo. = indomethacin.

Prostaglandin E₂, Production by Human PBMCs

Finally, we studied PGE₂ production by human PBMCs cocultured with glioma. As shown in Fig. 8A and B, U251 and T98G human glioma cells themselves produced only a small amount of PGE₂. However, PBMCs produced a large amount of PGE₂ when cocultured with glioma cells treated with or without LPS. Conditioned glioma medium also had a similar effect (Fig. 8C). These results suggested that the enhanced production of PGE₂ by macrophages cocultured with glioma is a general phenomenon.

Discussion

In this study, we considered PGE₂ to be one of the key factors in the regulation of immune responses against glioma and studied the mechanism of PGE₂ production in glioma-bearing states. We found that glioma cells did not produce as much PGE₂ as that shown in many previous reports. Interestingly, however, when PBMCs or macrophages and glioma cells were cocultured in vitro, PGE₂ production was markedly enhanced (Fig. 1). A cell culture insert did not disrupt PGE₂ production, and the conditioned glioma medium enhanced PGE₂ production, suggesting that the enhanced PGE₂ production by macrophages is mediated by glioma-derived soluble factors (Fig. 2). The conditioned glioma medium suppressed the macrophagic production of
TNF and IL-12, important cytokines for tumor immunity, and this suppression was abrogated by indomethacin, a COX inhibitor (Fig. 4). Furthermore, the conditioned glioma medium suppressed the induction of TNP-specific cytotoxic T cells, a model system for tumor antigen-specific T cells; this suppression was also overcome by indomethacin (Fig. 5).

The glioma-derived soluble factors found in this study are dialysis membrane–impermeable and sensitive to heat and papain treatment, suggesting that they are proteins (Fig. 3). We also found that several types of tumor cells, such as EL-4 thymoma, MH-134 hepatoma, and MBT-2 bladder cancer, also produce similar soluble factors (data not shown). Several reports have indicated that tumor cells produce many kinds of soluble factors such as TGFβ and cytokines. Among these cytokines, IL-1 and TNF are reported to stimulate macrophages to produce PGE2. Note, however, that the glioma cells studied in the present report did not produce these cytokines (Table 1). Furthermore, an-
and mPGES-1 is biosynthesis. Au-production by macrophages was not enhanced by reported reproduction production and the expression of COX-2 and impaired host production by macrophages in glioma-bearing hosts. Recently, production and Maeda, et al. stimulated macrophages, Type V, COX-2 and mPGES-1 are inducible enzymes, especially the expression of mPGES-1, was enhanced by glioma-derived soluble factors. There was no significant difference in the expression of cPLA2, sPLA2, Type V, COX-1, or cPGES/p23 after treatment with the glioma-derived soluble factors (Fig. 6). It is known that COX-2 and mPGES-1 are inducible enzymes, and mPGES-1 is proposed to be preferentially coupled with the inducible COX-2 to promote the final step of PGE2 biosynthesis. Reportedly, the induced expression of mPGES-1 is associated with various pathophysiological reactions, such as rheumatoid arthritis, febrile response, reproduction and bone metabolism disorders, and Alzheimer disease. Recently, it was suggested that the expression of mPGES-1 in combination with COX-2 contributes to tumorigenesis. Authors of these reports have indicated that PGE2 production by tumor cells is related to tumor cell growth by inhibiting apoptosis and by inducing angiogenesis. Therefore, much attention has thus far been focused on the expression of mPGES-1 and the production of PGE2 by tumor cells; conversely, there is little information about the expression of mPGES-1 and the production of PGE2 by macrophages. Based on our findings, we posited that glioma induces COX-2 and mPGES-1 in macrophages, which consequently produce an enhanced amount of PGE2, and that this induction is one of the mechanisms of immunosuppression in glioma-bearing hosts. Moreover, it has been reported that COX inhibitors have an antitumor effect against various tumor cells, including glioma, and that the mechanism of this effect is mainly considered to be an inhibition of COX-2 in tumor cells. However, our results indicated that the target of COX inhibitors may be tumor-infiltrating macrophages, or both macrophages and tumor cells. Furthermore, as shown in Fig. 7, mPGES-1 can be considered an important target for inducing an antiglioma effect given that COX inhibitors may be tumor-infiltrating macrophages, or both macrophages and tumor cells. Furthermore, as shown in Fig. 7, mPGES-1 can be considered an important target for inducing an antiglioma effect given that IL-12 production was not clearly suppressed in mPGES-1-deficient mice. Thus, selective mPGES-1 inhibitors will be a possible candidate for the induction of antiglioma effects in the future.

**Conclusions**

We found that glioma produced soluble factors that stimulated macrophages to produce PGE2 and impaired host immune responses. These soluble factors induced COX-2 and mPGES-1, especially the latter, in macrophages. This

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**TABLE 1**

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<th>Cytokine production by glioma cells*</th>
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<td>VM (pg/ml)</td>
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<td>RSV-M</td>
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<td>IL-12 (pg/ml)</td>
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* The VM or RSV-M glioma (2 × 10⁶/ml) was cultured for 48 hours, and cytokine production was detected using ELISA. Abbreviation: ND = under the detection limit.
mechanism is a new means of inducing an immunosuppressive state in glioma-bearing hosts. We consider the improvement of an immunosuppressive state to be required for the effective induction of antiglioma immune responses; therefore, the inhibition of PGE synthesis is one of the treatments for improving an immunosuppressive state. Although further characterization of the glioma-derived soluble factors and clarification of the precise mechanism of enhanced PGE production are required, we consider not only COX-2 but also mPGES-1 to be therapeutic targets for the antiglioma effect in the future.

References

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Address reprint requests to: Uki Yamashita, M.D., Ph.D., Department of Immunology, School of Medicine, University of Occupational and Environmental Health, Japan, 1-1 Iseigaoka, Yahatanishiku, Kitakyushu 807-8555, Japan. email: yama-uki@med.uoeh-u.ac.jp.