Different Progression of Tumor Xenografts between Mucin-Producing and Mucin–Non-Producing Mammary Adenocarcinoma-Bearing Mice

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Abstract

Previously, we found that MUC2 mucins could activate monocytes/macrophages through a scavenger receptor leading to cyclooxygenase (COX) 2 induction and overproduction of prostaglandin E2 (PGE2). To investigate the role of mucins in the tumor-bearing state, we compared s.c. tumor formation by using mucin-producing (TA3-Ha) and mucin–non-producing (TA3-St) cloned variants of mouse mammary adenocarcinomas. Expression of COX2 mRNA and protein and production of PGE2 were elevated in peritoneal macrophages stimulated with epiglycanin, which is a mucin-like glycoprotein produced by TA3-Ha cells. S.c. tumor tissues comprising TA3-Ha cells grew much faster than tissues comprising TA3-St cells. COX2 protein and vascular endothelial growth factor in TA3-Ha tumor tissues were elevated compared with the TA3-St tumor tissues. Although similar numbers of macrophages were observed immunohistochemically in the two types of tumor tissues, COX2 was induced prominently in the infiltrating macrophages in TA3-Ha tumor tissues but only faintly in TA3-St tumor tissues. Furthermore, angiogenesis progressed more rapidly in TA3-Ha tumor tissues but only slightly in TA3-St tumor tissues. Epiglycanin-induced overproduction of PGE2 down-regulated interleukin-12 production by macrophages. IFN-γ-producing CD4 T cells in spleens obtained from TA3-Ha tumor-bearing mice were significantly reduced compared with TA3-St tumor-bearing mice, suggesting that mucins cause PGE2-mediated immune suppression. Actually, the tumor growth of a TA3-Ha cell xenograft was suppressed effectively by oral administration of a COX2 inhibitor but that of a TA3-St cell one was not. These results suggest that mucins play an important role in tumor progression through overproduction of PGE2. (Cancer Res 2006; 66(12): 6175-82)

Introduction

Numerous studies on rodent cancer models and human cancers have shown that nonsteroidal anti-inflammatory drugs (NSAID) have antineoplastic properties. NSAIDs are known to inhibit cyclooxygenase (COX; ref. 1). Two isoforms of COX (i.e., COX1 and COX2, which are constitutive and inductive enzymes, respectively) have been identified (2, 3). Expression of COX2 is up-regulated in various types of cancer (4–6). Recent studies have led to the recognition of the importance of COX2 in colorectal tumorigenesis. Williams et al. (7) reported that host COX2 modulated carcinoma growth. Oshima et al. (8) obtained direct evidence that the formation of intestinal polyps in ApcΔ716 knockout mice was dramatically suppressed by crossing them with COX2 knockout mice. These reports indicate that COX2 acts as a rate-limiting agent and contributes at an early stage of carcinogenesis. They also showed that COX2 is not expressed in colon epithelial cells but in interstitial cells at an early stage. There have been some reports showing the expression of COX2 in infiltrating macrophages in tumor tissues (6, 9).

It is well known that prostaglandin E2 (PGE2) has various biological effects, such as inhibition of apoptosis (10, 11) and immunosurveillance (12, 13) and promotion of tumor angiogenesis (14–17) and invasion (18–20). Thus, despite much evidence that COX2 overexpression is crucial for tumorigenesis and tumor growth, the mechanism by which COX2 is induced remains unresolved. Recently, we showed that mucins secreted from colon cancer cells could induce COX2 in monocytes/macrophages (9).

Many tumors arising from epithelial tissues produce mucins. They are characterized by their O-glycosylated domains, which contain a repetitive backbone with especially high contents of threonine and serine residues. On malignant transformation, many epithelial cells produce mucins in abnormal amounts and/or with abnormal glycosylation patterns (21). Mucins produced by cancer cells are found in the sera of cancer patients and are used as disease markers. It has been reported that cancer patients with higher amounts of mucins in their bloodstream have a lower 5-year survival rate (22). However, little is known regarding the biological significance of mucins. Mucins readily come into contact with various cells circulating in the bloodstream in cancer patients and/or with the infiltrated cells in cancer tissues.

In the present study, we aimed to determine to what extent mucins affect the formation of tumor tissues and the immune system in tumor-bearing mice through overproduction of PGE2. A survey of mouse epithelial cancer cells revealed that mouse mammary adenocarcinoma cell lines TA3-Ha and TA3-St may be a closely matched pair that could be exploited to compare tumor growth and the immunologic state in relation to mucins, because TA3-Ha cells produce a mucin named epiglycanin, but TA3-St cells, a subline, do not (23). Epiglycanin is a sialylated, membrane-associated glycoprotein with a large mucin-like domain protruding 500 nm above the cell surface (24). Carbohydrate moieties comprise 75% to 80% of the molecule by weight, essentially all of which comprises short O-glycans. Because of its length and high level of expression, epiglycanin has been suggested to have an immunosuppressive effect, including masking of cell surface molecules, such as class I MHC antigen (25–27). Epiglycanin is
shed from the cell surface and can be detected in the ascites fluid and in the serum of mice.

In the present study, we show that epiglycanin could elevate PGE₂ production through COX2 induction in mouse macrophages, which was very advantageous for tumors due to the advanced angiogenesis and immune suppression. In fact, oral administration of a COX2 inhibitor suppressed s.c. tumor growth of TA3-Ha cells effectively but did not suppress that of TA3-St cells. Thus, we postulate that mucins facilitate tumor progression in a tumor microenvironment.

Materials and Methods

Cells, mice, and materials. The two mouse mammary adenocarcinoma sublines (TA3-Ha and TA3-St) were kindly provided by Dr. Hilkens (National Cancer Institute of Netherlands). The cells were maintained in ascites fluid by i.p. inoculation into syngeneic strain A mice. A/J mice (7-9 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mouse monoclonal antibody (mAb) MLS12 recognizing Tn antigen was prepared as described previously (28). Bovine submaxillary mucin (BSM) was obtained from Roche Diagnostics (Basel, Switzerland). A COX2 inhibitor, Etodolac, was kindly provided by Nippon Shinyaku Co. Ltd. (Kyoto, Japan).

Tumor growth. TA3-Ha or TA3-St cells (1 \( \times 10^6 \)) in 0.1 mL PBS were implanted s.c. into the backs of mice, and the tumor growth was determined by measuring the tumor volume. When examining the effect of a COX2 inhibitor, oral administration of Etodolac (10 mg/kg) or the drug vehicle (0.1 mL of 40% ethanol) was done daily after inoculation.

Preparation of mouse peritoneal macrophages and splenic CD4 T cells. At 3 days after i.p. injection of 3% thioglycollate, peritoneal exudate cells were obtained by peritoneal lavage with ice-cold HBSS and then washed twice with HBSS, from which macrophages were isolated using anti-mouse CD11b mAb (rat, IgG2b) microbeads and MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Splenocytes were derived from normal A/J mice and then passed through a sterile stainless mesh with a rubber stopper. After lysis of RBC by treatment with 0.17 mol/L NH₄Cl on ice, the cells were washed with RPMI 1640, from which CD4 T cells were isolated using anti-mouse CD4 mAb (rat, IgG2b) microbeads and MACS system according to the manufacturer's instructions. These cells were cultured in RPMI 1640 supplemented with 10% FCS.

Isolation of epiglycanin from extracts of TA3-Ha tumor tissues. Mouse mammary adenocarcinoma TA3-Ha cells (1 \( \times 10^6 \)) were injected s.c. into mice. After 3 weeks, tumor tissues were removed and extracted with 25 mmol/L Tris-HCl (pH 7.5), 2 mmol/L EDTA, and 0.15 mol/L NaCl. The extracts were subjected to gel filtration on Sephacryl S-200 (4 \( \times 100 \) cm). Fractions of 14 mL were collected, and the absorbance at 254 nm was determined. Each fraction was added to the culture medium of normal peritoneal macrophages (1\( \times 10^6 \)), which was very advantageous for tumors due to the advanced angiogenesis and immune suppression. In fact, oral administration of a COX2 inhibitor suppressed s.c. tumor growth of TA3-Ha cells effectively but did not suppress that of TA3-St cells. Thus, we postulate that mucins facilitate tumor progression in a tumor microenvironment.

Western blot analysis of COX2 protein in mouse peritoneal macrophages and splenic CD4 T cells. After 6 hours with epiglycanin (1.0 μg protein/mL) or BSM (30 μg protein/mL) and solubilized with 50 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 0.15 mol/L NaCl, 1 mmol/L EDTA, pepstatin A (1 μg/mL), and leupeptin (1 μg/mL), and COX2 protein and β-actin were immunoprecipitated with anti-mouse COX2 antibody (goat, IgG; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-mouse β-actin antibody (clone AC-15, IgG1; Sigma-Aldrich, respectively).

Determination of vascular endothelial growth factor by ELISA. The two mouse mammary adenocarcinoma TA3-Ha or TA3-St tumor tissues. Mouse peritoneal macrophages (1 \( \times 10^6 \)) were treated for 6 hours with epiglycanin (1.0 μg protein/mL) or BSM (30 μg protein/mL) and solubilized with 50 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 0.15 mol/L NaCl, 1 mmol/L EDTA, pepstatin A (1 μg/mL), and leupeptin (1 μg/mL), and COX2 protein and β-actin were immunoprecipitated with anti-mouse COX2 antibody (goat, IgG; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-mouse β-actin antibody (clone AC-15, IgG1; Sigma-Aldrich, respectively).

Histological detection of epiglycanin, Mac1, COX2, and CD31. At 5 days after s.c. inoculation (1 \( \times 10^6 \)) of TA3-Ha and TA3-St cells, tumor
tissues were removed, and frozen serial tissue sections were cut into 10-μm slices and fixed with 4% paraformaldehyde. The samples were incubated with 5% BSA-PBS at room temperature for 1 hour to block nonspecific binding. For detection of Tn antigen using mouse mAb MLS 128, the sample was treated with a Histo Mouse Plus kit (Zymed Laboratories, Inc., San Francisco, CA) to block endogenous IgG. The specimen was incubated with primary antibodies, anti-mouse Mac1 antibody (goat, IgG; Santa Cruz Biotechnology), anti-mouse COX2 antibody, MLS 128, or anti-mouse CD31 antibody (rat, IgG2a; BD Biosciences, San Jose, CA) at room temperature for 30 minutes and then with each secondary antibody conjugated with rhodamine or FITC. As a negative control, the primary antibodies were omitted during the staining procedure.

Results

Proliferation of TA3-Ha and TA3-St cells in vitro and in vivo. First, we compared the proliferation of TA3-Ha and TA3-St cells in vitro and in vivo. The two sublines showed similar growth curves in vitro (Fig. 1A). In contrast, when injected s.c. into mice, the TA3-Ha tumor grew much faster than the TA3-St one did (Fig. 1B). The different growth rates of these cell sublines may be relevant to the biological function of epiglycanin, including the fact that epiglycanin functions as a barrier against immune attack as reported previously (25, 26).

Isolation of epiglycanin from TA3-Ha tumor tissues and its ability to elevate PGE2 production by mouse peritoneal macrophages. Previously, we found that conditioned medium of a colon cancer cell line, LS 180, contained a factor that induces COX2 in human peripheral blood monocytes. The factor was purified and revealed to be mucins. The MUC2 mucins induced COX2 mRNA and caused overproduction of PGE2 in dose- and time-dependent manner (9). To further investigate the biological function of these mucins, we tried to isolate epiglycanin from TA3-Ha tumor tissues. Extracts of tumor tissues were fractionated by gel filtration on Sepharose 6B. Protein (1 μg) of each fraction was added to the culture medium of mouse peritoneal macrophages. As shown in Fig. 2A, the excluded fractions remarkably elevated the production of PGE2. A Tn antigen expressed on the core protein of epiglycanin was detected in the same fractions on dot-blot analysis (Fig. 2B). From the excluded fractions, epiglycanin was purified according to Baeckstrom et al. (29). The purity of the epiglycanin was examined by SDS-PAGE followed by Western blotting. Expression of the Tn antigen was confirmed (Fig. 2C, lane c).

Purified epiglycanin or BSM was added to the culture medium of mouse peritoneal macrophages followed by culturing for 20 hours, and supernatants were collected for the determination of PGE2 production by ELISA. As shown in Fig. 3A, both epiglycanin and BSM enhanced PGE2 production in a dose-dependent manner. A small amount of epiglycanin (0.5 μg protein/mL) or BSM (0.3 μg protein/mL) could induce the production of PGE2.

To determine whether epiglycanin- or BSM-induced production of PGE2 was associated with up-regulation of COX2, COX2 mRNA in macrophages was detected after treatment with epiglycanin or BSM. Macrophages were incubated with various concentrations of epiglycanin or BSM for 4 hours, total RNA was prepared, and RT-PCR was done. A corresponding amount of epiglycanin or BSM, as described above, induced COX2 mRNA but not COX1 mRNA (Fig. 3B). Induction of COX2 protein in macrophages after treatment with epiglycanin or BSM for 6 hours was also confirmed by Western blot analysis as shown in Fig. 3C. The results indicate that PGE2 production was enhanced through a COX2-mediated pathway. Incubation of macrophages in the presence of indomethacin (0.1 μmol/L) reduced the production of PGE2 to the control level (data not shown).

COX2 induction and angiogenesis in TA3-Ha and TA3-St tumor tissues. TA3-Ha or TA3-St cells (1 × 106) were injected s.c. into mice. After 5 days, tumor tissues were removed and proteins were extracted from the tumor tissues. Epiglycanin and COX2 protein were immunoprecipitated from the extracts and the immunoprecipitates were subjected to SDS-PAGE followed by Western blotting. Epiglycanin was detected in the extracts of TA3-Ha tumor tissues but not in those of TA3-St tumor tissues as described previously (ref. 23; Fig. 4A). COX2 protein was also detected in TA3-Ha tumor tissues but only very slightly in TA3-St tumor tissues (Fig. 4A).

It has been reported that PGE2 enhances the expression of VEGF (14, 32). VEGF was determined by ELISA. As shown in Fig. 4B, VEGF was clearly elevated in TA3-Ha tumor tissues compared with TA3-St tumor tissues.

Next, we examined TA3-Ha and TA3-St tumor tissues immunochemically. Tumor tissues were removed as described above, fixed with 4% paraformaldehyde, and then observed immunochemically (Fig. 4C). Similar numbers of macrophages had infiltrated into...
the tumor tissues of TA3-Ha and TA3-St tumor-bearing mice, and COX2 was detectable in TA3-Ha tumor tissues but only very slightly in TA3-St tumor tissues. Interestingly, the merged macrophage and COX2 indicates that COX2 was induced in most infiltrating macrophages in TA3-Ha tumor tissues. The other cells around the macrophages also expressed COX2 weakly, the expression probably being induced by mediators secreted from activated macrophages. It should also be noted that CD31+ cells were clearly observed in TA3-Ha tumor tissues but only slightly in TA3-St tumor tissues, indicating advanced angiogenesis in TA3-Ha tumor tissues.

**Immunologic effect of epiglycanin on production of Th1 cytokines.** A tumor-bearing state induces an abnormal cytokine network through which the production of antitumor cytokines is negatively regulated. Tumor and/or host products have been implicated in the impairment of immune responses in various systems (33, 34). Soluble factors produced by tumor cells could be the most plausible explanation for the systemic effect of a localized tumor. PGE2 is a well-known immunomodulator that has multiple effects on the immune system (35, 36). Although IL-12 production by several cell types has been reported, macrophages and dendritic cells are believed to be the main source of this cytokine (37). It has been reported that PGE2 suppresses LPS-induced IL-12 production by macrophages and dendritic cells (35). To see if PGE2 production induced by epiglycanin or BSM causes down-regulation of IL-12, peritoneal macrophages were pretreated with epiglycanin (2 μg protein/mL) or BSM (30 μg protein/mL) for 20 hours, and the cells were stimulated with LPS (0.1 μg/mL). The levels of IL-12 produced after 18 hours were determined by ELISA. As shown in Fig. 5A, treatment of macrophages with epiglycanin or BSM resulted in

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**Figure 2.** Isolation of epiglycanin from extracts of TA3-Ha tumor tissues. A, extracts of tumor tissues were prepared as described in Materials and Methods, subjected to gel filtration on Sepharose 6B (4 × 100 cm), and then eluted with 25 mmol/L Tris-HCl (pH 7.5), 2 mmol/L EDTA, and 0.15 mol/L NaCl. Fractions of 14 mL were collected, and the absorbance at A280 (•–•) was determined. Protein (1 μg) of each fraction was added to the culture medium of normal peritoneal macrophages (1 × 106). After 24 hours, PGE2 secreted was determined by ELISA. B, each fraction (100 μL) was loaded on a nylon membrane and dot-blot analysis was done using mAb MLS 128. C, purified epiglycanin (5 μg protein) was subjected to SDS-PAGE followed by Coomassie brilliant blue (lane a) and periodate-Schiff (lane b) staining. Another sample was transferred to a nylon membrane, and Tn antigen borne on the core protein was detected with MLS 128 (lane c). Epiglycanin apparently remained near the top of the 6% running gel (arrow).
significant inhibition of IL-12 production, indicating that enhanced production of PGE2 in macrophages induced by mucins has a suppressive effect on IL-12 production by macrophages as an autocrine mechanism. Incubation of macrophages in the presence of indomethacin (0.1 μmol/L) almost restored the production of IL-12 (data not shown).

TA3-Ha or TA3-St cells (1 × 10⁶) were implanted s.c. After 3 weeks, splenic T cells were prepared as described in Materials and Methods and then cultured in the presence or absence of concanavalin A (5 μg/mL). After 20 hours, the culture medium was collected and the level of IFN-γ was determined by ELISA. As shown in Fig. 5B, concanavalin A–stimulated T cells obtained from TA3-Ha tumor-bearing mice produced significantly reduced levels of IFN-γ compared with T cells from control or TA3-St tumor-bearing mice. Unstimulated T cells from either control mice or tumor bearers did not produce a detectable level of IFN-γ (data not shown).

To determine whether the number of INF-γ-producing T cells is altered in the TA3-Ha tumor-bearing state, splenic CD4 T cells in which IFN-γ was pooled intracellularly through pretreatment with brefeldin A were analyzed. TA3-Ha cells, TA3-St cells, or PBS, as a control, were injected s.c. into mice. After 3 weeks, splenic CD4⁺ T cells were prepared and treated as described in Materials and Methods. As shown in Fig. 5C and D, although a small percentage of T cells produced IFN-γ or IL-4, IFN-γ-producing T cells in TA3-Ha tumor-bearing mice were significantly reduced compared with control mice. IFN-γ-producing T cells in TA3-St tumor-bearing

Figure 4. COX2 induction and angiogenesis in TA3-Ha and TA3-St tumor tissues. A, epiglycanin, COX2 protein, and β-actin were immunoprecipitated from extracts of TA3-Ha and TA3-St tumor tissues, and the precipitates were subjected to SDS-PAGE followed by Western blotting. Each protein was detected as described in Materials and Methods. B, VEGF extracted from TA3-Ha and TA3-St tumor tissues was determined by ELISA. *, P < 0.05 versus TA3-St. C, TA3-Ha and TA3-St tumor tissues were prepared, fixed, and then immunostained as described in Materials and Methods. Mac1 and CD31 antigens show the distribution of macrophages and blood vessels, respectively. The merged Mac1 + COX2 indicates COX2 induced in macrophages. Tn antigen is expressed on epiglycanin.

Figure 5. Immunologic effect of epiglycanin on production of Th1 cytokines. A, peritoneal macrophages (1 × 10⁶) from normal mice were cultured for 20 hours in the presence of epiglycanin (2 μg protein/mL) or BSM (30 μg protein/mL), and the cells were stimulated with LPS (100 ng/mL). Cell-free supernatants were collected after 18 hours, and IL-12 levels were determined by ELISA. Columns, average (n = 3); bars, SD. *, P < 0.05; **, P < 0.01 versus control. B, splenic T cells from mice with a TA3-Ha or TA3-St tumor burden for 3 weeks were prepared as described in Materials and Methods. Cells (1 × 10⁶) were cultured in the presence or absence of concanavalin A (5 μg/mL) for 20 hours. Cell-free supernatants were collected, and IFN-γ levels were determined by ELISA. Columns, average (n = 4); bars, SD. *, P < 0.01 versus control. C, splenic CD4 T cells prepared as described above were treated with phorbol 12-myristate 13-acetate, ionomycin, and brefeldin A as described in Materials and Methods. After fixation and saponin treatment, specimens were analyzed with a flow cytometer. Columns, average (n = 6); bars, SD. *, P < 0.05 versus PBS-IFN-γ.
mice were also reduced to a lesser extent. An inverse relationship between the levels of Th1 and Th2 cytokines has been well documented (38). However, the number of IL-4-producing T cells did not differ between control and tumor-bearing mice.

Treatment of tumor-bearing mice with a COX2-specific inhibitor, Etodolac, in vivo. To determine to what extent mucin-mediated PGE2 production is related to tumor growth, tumor-bearing mice were treated with a COX2 inhibitor in vivo. As shown in Fig. 6, the tumor growth of TA3-Ha cells was effectively suppressed by treatment with Etodolac, but the tumor growth of TA3-St cells was not.

Discussion

It has not been clearly elucidated how tumor and/or host products lead to favorable conditions for tumor growth in a tumor microenvironment. Angiogenesis, invasion, and defense from the immunity of the host may be essential for tumor progression. It is well known that PGE2, one of the factors produced by a host and/or tumor, plays critical roles in diverse biological processes, including immune suppression (12, 13) angiogenesis (14–17), and invasion (18–20). Previously, we reported that mucins produced by epithelial cancer cells could bind to a scavenger receptor of monocytes leading to overproduction of PGE2 through the COX2-mediated pathway (9). In addition, mucins in the bloodstream of patients with epithelial cancer actually induced COX2 and enhanced the production of PGE2 by peripheral blood monocytes, suggesting that overproduced PGE2 may be relevant to various biological processes, such as immune suppression (39).

To further evaluate the role of mucins in a tumor-bearing state, we did the present study using mice bearing a mucin-producing or mucin–non-producing mouse mammary adenocarcinoma cloned variant. Epiglycanin is present on the cell surface of TA3-Ha cells as described previously (24). Thingstad et al. reported that fully mature epiglycanin is shed from the cell surface, its half-life being ~60 hours (40). Thus, in addition to direct contact with epiglycanin on the cell surface, shed epiglycanin may interact with host cells in tumor tissues and/or the bloodstream. On the gel filtration of tumor extracts, the ability to enhance PGE2 production by macrophages was detected for the excluded fractions corresponding to epiglycanin-eluted fractions (Fig. 2). It seems unlikely that the activity is due to other proteins, such as cytokines and growth factors, which may be associated with epiglycanin, because these excluded fractions retained the activity even after heat treatment. As discussed later, epiglycanin activated macrophages through a scavenger receptor, which recognizes anionic charges on carbohydrate moieties but not the core protein. Therefore, the ligand activity of epiglycanin was not abolished on heat treatment. When purified epiglycanin was added to the culture medium of mouse peritoneal macrophages, induction of COX2 mRNA and production of PGE2 were both elevated in a dose-dependent manner (Fig. 3).

Next, we tried to determine whether the elevated PGE2 production induces the expression of VEGF. There have been many reports that overexpression of COX2 in tumor tissues may be important in tumor invasion and angiogenesis. As expected, VEGF production was elevated in TA3-Ha tumor tissues compared with TA3-St tumor tissues. Recently, Chang et al. (15) showed that PGE2 induced angiogenesis at the earliest stage of tumor development. The mechanism by which COX2 is induced by mucins produced by tumors may provide, in part, the molecular basis of how COX2 is induced from an early stage of tumor development.

Immunohistochemical studies confirmed that COX2 was induced in infiltrating macrophages in TA3-Ha tumor tissues but not in macrophages in TA3-St tumor tissues, suggesting mucin-mediated COX2 induction. These results are consistent with our previous finding that COX2 was only induced in macrophages that had infiltrated around the region with expression of mucins in human colorectal cancer tissues (9). In TA3-Ha tumor tissues, COX2 expression was also observed in other cells. Sonoshita et al. (41) showed that COX2 expression is enhanced by PGE2 through the EP2 receptor via a positive feedback loop. Thus, once PGE2 is produced by macrophages, it exerts a positive effect, inducing the expression of COX2 in its own position and/or nearby cells, resulting in a substantial increase in the capacity of tissues to synthesize and release PGE2. These results are consistent with the reports describing the expression of COX2 in infiltrating macrophages in tumor tissues (6, 9) and elevated PGE2 release from macrophages in tumor-bearing mice (42).

It is also generally agreed that overexpression of COX2 in tumor tissues may be related to immune suppression (18, 19). When normal macrophages were pretreated with epiglycanin (2 μg protein/mL) or BSM (30 μg protein/mL), LPS-induced production of IL-12 (p40) by macrophages themselves was reduced to ~50% and 10% of the control level, respectively. This difference in the level of reduction between epiglycanin and BSM may be due to different levels of PGE2 produced by macrophages under these conditions as shown in Fig. 3. These results are consistent with the report that PGE2 is capable of decreasing the level of IL-12 produced by normal macrophages (43). Spent medium of DA3 mouse mammary tumor cells also inhibited the production of IL-12 by normal macrophages, which is considered to be due to tumor-derived PGE2 and phosphatidylyserine (43).

It has also been reported that antigen-presenting cell–derived PGE2 down-regulates IFN-γ production by T cells (38) and that IL-12 induces IFN-γ production by T and natural killer cells (44, 45). IFN-γ production by splenic CD4 T cells from TA3-Ha
In contrast, splenic CD4 T cells from TA3-St tumor-bearing mice produced a slightly lower level of IFN-γ, suggesting that epiglycanin may be related to IFN-γ production through PGE₂ production. We also compared IFN-γ- and IL-4-producing T cells in spleens among control mice and TA3-Ha and TA3-St tumor-bearing mice. It should be noted that IFN-γ-producing cells were definitely reduced in CD4 T cells from TA3-Ha tumor-bearing mice compared with cells in control or TA3-St-bearing mice, suggesting that tumor-derived mucins may be responsible for the decreased IFN-γ production through PGE₂ production by macrophages from tumor bearers (Fig. 5C and D). Whereas IL-4-producing cells did not show any change irrespective of whether they were from control or tumor-bearing mice. These results are consistent with the report that the synthesis of Th1 cytokines is much more sensitive to inhibition by PGE₂ than Th2 cytokine production (46). For in vitro experimental systems involving human cord blood as a source of naive lymphocytes, it should be considered that small amounts of IL-4 and IFN-γ can be detected at the time of the first stimulation and that they profoundly affect the ability of cells to produce IL-4 and IFN-γ, respectively (47). These experiments suggested that PGE₂ might exert its effect by inhibiting the production of the small amount of IFN-γ at the time of priming. Similar conditions may be produced by mucins secreted from tumor cells, this being consistent with the report of gradual loss of Th1 populations in the spleens of mice during progressive tumor growth (34). This immunologic suppression and promotion of angiogenesis may collectively facilitate the progression of TA3-Ha xenografts. To further study the relationship between mucins and tumor progression, tumor-bearing mice were treated with a COX2 inhibitor. The COX2 inhibitor suppressed effectively the growth of TA3-Ha xenografts but not that of TA3-St xenografts. These results suggest that mucin-mediated COX2 induction may be related to promotion of TA3-Ha tumor progression through PGE₂ overproduction.

Because peritoneal macrophages are activated by both epiglycanin and BSM, it seems that the binding sites are carbohydrate moieties but not the core protein. Previously, we found that peripheral blood monocytes recognize mucins through a scavenger receptor (9). It has been reported that the scavenger receptor could recognize a pattern of anionic charges on ligand molecules, such as acetyl low-density lipoprotein, poly I, fucoidan (48), and LPS (49). Anionic charges due to sialic acid and sulfate borne on O-glycans of mucins may be recognized by the receptor. To examine the binding of epiglycanin and BSM to the scavenger receptor, we prepared a recombinant scavenger receptor possessing an ectodomain (group A, type I) and confirmed the binding of the receptor to epiglycanin and BSM by a plate assay. However, because mucins have a variety of O-glycans, there remains the possibility that other receptors may recognize mucins.

Overall, we propose the following cascade in the tumor-bearing state. Mucins are produced by cancer cells. Infiltrating macrophages are activated by the mucins through the scavenger receptor. PGE₂ secreted from the macrophages binds to the EP2 receptor present on cancer cells and/or other cells as reported by Sonoshita et al. (41). PGE₂ secreted from various cells up-regulates VEGF production and down-regulates IL-12 production by macrophages in an autocrine manner and IFN-γ production by Th1 cells in a paracrine manner, leading to the promotion of angiogenesis and the prevention of rejection of a tumor.

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