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Differential Expression and Regulation of Cyclooxygenase Isozymes in Thymic Stromal Cells

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Prostaglandins (PGs) are lipid-derived mediators of rapid and localized cellular responses. Given the role of PG in supporting thymic T cell development, we investigated the expression of the PG synthases, also known as cyclooxygenases (COX)-1 and -2, in the biosynthesis of PGs in thymic stromal cell lines. The predominant isozyme expressed in cortical thymic epithelial cells was COX-1, while COX-2 predominated in the medulla. IFN-γ up-regulated expression and activity of COX-2 in medullary cells, in which COX-2 was expressed constitutively. In contrast, IFN-γ down-regulated COX-1 activity, but not expression, in cortical cells. Stromal cells support T cell development in the thymus, although the mediators of this effect are unknown. Selective inhibition of COX-2, but not COX-1, blocked the adhesion of $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes to medullary cell lines. No effect of the inhibitors was observed on the interactions of thymocytes with cortical epithelial lines. These data further support the differential regulation of COX-1 and COX-2 expression and function in thymic stromal cells. PGs produced by COX-2 in the medullary thymic stroma may regulate the development of thymocytes by modulating their interaction with stromal cells. ***The Journal of Immunology,*** **1999, 162: 4589–4597.**

Prostaglandins (PGs) are evanescent biologically active mediators (1). Their formation from arachidonic acid (AA)[2] is catalyzed by PG G/H synthases, colloquially known as cyclooxygenases (COX) (1). Two COX isoforms have been identified (2–4). In general terms, COX-1 subserves a "housekeeping" function and it is widely expressed constitutively (1). Expression of COX-2 is generally regulated by cytokines, tumor promoters, and growth factors (5). Despite these observations, there are examples of regulated expression of COX-1 (1), while COX-2 is expressed constitutively in certain regions of the brain, the reproductive system, and the kidney (6). COX-2 is also likely to be physiologically up-regulated by shear stresses exerted on the vascular endothelium (7, 8). The concept that COX-2 generally accounts for enhanced PG formation in foci of inflammation and in developing tumors underlies the development of selective inhibitors of this isozyme (5, 6, 9).

Increasing evidence supports the hypothesis that eicosanoids modulate the immune response mediated by mature T cells, as well as T cell development within the thymus. For example, PGE_2 modulates cytokine secretion by both human and murine mature $CD4^+$ T cells. This eicosanoid, which is synthesized by APCs, such as macrophages and fibroblasts, inhibits Th1 cytokines, such as IL-2, whereas it enhances production of Th2 cytokines, such as IL-4 and IL-5 (10–15). PGE_2 also inhibits the adhesion and motility of cytotoxic T lymphocytes (16). Synthesis of PGE_2 is increased in clinical syndromes of immune disfunction, such as AIDS (17, 18), autologous bone marrow and stem cell transplantation (19), atopic dermatitis, and the hyper IgE syndrome (20).

Aside from the effects of PGs within the mature T cell compartment, they may also influence maturation of the T cell lineage. Thus, thymic expression of various PG biosynthetic enzymes and receptors has been detected in human (21–24), mouse (23–25), and rat (26) thymus. Furthermore, thymus and nonlymphoid thymic stromal cell lines have been shown to secrete PGs in vitro (27–29). While PGE_2 protects a human thymocyte cell line from apoptosis (30), it has also been reported to initiate apoptosis in neonatal primary thymocytes (31). More recently, using COX-deficient mice and pharmacological inhibitors, we have found that COX-1-dependent PGE_2 formation facilitates the transmission of $CD4^-CD8^-$ double-negative (DN) thymocytes to $CD4^+CD8^+$ double-positive (DP) cells, acting via the EP2 subtype of the PGE_2 receptors (32). By contrast, COX-2-dependent PGE_2 formation supports $CD4^+$ single-positive (SP) lymphocyte selection and earlier stages of thymocyte differentiation, in this case acting via the EP1 subtype of the PGE_2 receptor (32).

Differentiation and migration of thymocytes occurs within a complex cellular network known as the thymic stroma (33). Among the constituents of the stroma are nurse cells, cortical reticular epithelial cells, medullary epithelial cells, nonepithelial dendritic cells, and macrophages (33, 34). It is thought that each of these cell types subserves distinct functions in thymocyte development, but the molecular bases of these cellular interactions are poorly understood. Cortical epithelial cells are thought to influence positive selection, while medullary epithelial cells appear to contribute to induction of tolerance and to the later stages of thymocyte development (33, 34).

Given our observations in the fetal thymus, where the spatial expression and functional roles of the COX isozymes are distinct (32), we wished to examine the potential role of PGs in mediating

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[2] Abbreviations used in this paper: AA, arachidonic acid; COX, cyclooxygenase; DN, $CD4^-CD8^-$ double-negative thymocytes; DP, $CD4^+CD8^+$ double-positive thymocytes; SP, single-positive thymocytes; TX, thromboxane; EP, prostaglandin E receptors.

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interactions between thymocytes and stromal cells. We have used the role of IFN-γ to regulate the expression of COX isozymes in cell lines representative of cortical nurse (427.1), cortical reticular (1308.1), medullary interdigitating-like (6.1.1), and medullary epithelial (1307) cells in the thymic stroma (35). We report differential expression of the COX isozymes in cortical vs medullary cells. COX-1 expression is greatest in cortical cell lines. COX-2, by contrast, is constitutively expressed predominantly in medullary cells. IFN-γ up-regulates COX-2 and consequent production of PGE_2 in medullary cells, while it reduces COX-2 protein expression and PGE_2 production in cortical cell lines. Selective inhibition of COX-2, but not COX-1, prevents adhesion of DP and CD4 SP thymocytes to medullary, but not to cortical stromal cells, an effect which is reversed by PGE_2. These results suggest that COX-2-dependent PGE_2 formation in the medulla may influence selective interactions between developing thymocytes and constituents of the thymic stroma.

Materials and Methods

Materials

Recombinant mouse IFN-γ was obtained from PharMingen (San Diego, CA). AA and PGE_2 were purchased from Cayman Chemical (Ann Arbor, MI). NS-398 was purchased from Biomol (Plymouth Meeting, PA). L-759,700 was a generous gift of Dr. W. Tanaka (Merck). Nonidet P-40, indomethacin, BSA, and deoxycholic acid were bought from Sigma (St. Louis, MO). Tween 20 was obtained from J. T. Baker (Phillipsburg, NJ). Enhanced chemiluminescence (ECL) substrates and Hyperfilm ECL were obtained from Amersham (Arlington Heights, IL). Donkey polyclonal anti-mouse or anti-rabbit IgGs coupled to fluorescein or peroxidase were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). Anti-$CD4^-$FITC and anti-$CD8^-$Red-613 were obtained from Life Technologies (Gaithersburg, MD). C57BL and RAG-$1^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, MA) and maintained under standard conditions. Anti-mouse E-cadherin Ab was obtained from Transduction Laboratories (Lexington, KY). Anti-mouse-ICAM-1, CD40, CD44 Abs, and hyaluronate-fluorescein-conjugated Abs (36, 37) were a generous gift from Dr. Ellen Puré (The Wistar Institute, Philadelphia, PA). Anti-mouse β actin was obtained from Sigma.

Cell biology

Cells were cultured in DMEM, supplemented with 10% FCS, 2 mM L-glutamine, 5×10^{-5} M 2-ME, and 1 mM HEPES buffer. Confluent cells were washed in PBS and incubated with different concentrations of rIFN-γ added to the culture medium. Cells were collected at selected times and proteins were extracted as described below. Confluent cells were washed twice with HBSS containing 1 mg/ml BSA and incubated for 40 min in 4 ml of the same buffer with 10 μM AA to evaluate COX activity. Supernatants were collected for measurements of PGs. Cells were harvested by trypsinization and counted. The experiments were always performed in duplicate. Confluent cells were incubated with increasing concentrations of IFN-γ to study COX activity for 18–20 h, washed with HBSS, and stimulated with AA.

In experiments performed with COX inhibitors, cells were incubated for 40 min with L-759,700, NS-398, or indomethacin. Indomethacin was solubilized in absolute ethanol; NS-398 and L-759,700 were prepared in DMSO. Aliquots from each stock solution were added to fresh medium, and each preparation contained $\leq$0.1% ethanol or DMSO (v/v, final concentration), as did the control medium. Cells were then washed with HBSS/BSA and incubated for 40 min in the same buffer with 10 μM AA as already described.

Western blot analyses

Adherent cells were washed twice with PBS and lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 1 mM PMSF) for 15 min. Cells were scraped with a rubber policeman and centrifuged at 4°C for 10 min at $10{,}000 \times g$. The protein content was determined using a microbicinchoninic acid assay (Pierce, Rockford, IL) using BSA as standard. Cell lysates (40–50 μg of protein) were mixed with Laemmli reagent under reducing conditions. SDS-PAGE was performed according to standard techniques using 10% bis-acrylamide for the separation gel and transfer of proteins (38, 39). Nitrocellulose membranes were saturated for 2 h at room temperature in 5% fat-free dry milk-Tris buffer saline (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, and 0.1% Tween 20) (TBTT). Membranes were further incubated at room temperature for 1 h with specific mAbs for COX-1 (1:1000), COX-2 (1:2000) (kindly provided by the late Dr. J. Maclouf, Institut National de la Santé et de la Recherche Médicale Unit 348, Paris, France), or β actin (1:5000), or with polyclonal Abs directed against E-cadherin (1:1000). Blots were washed three times with TBTT buffer and then incubated with anti-rabbit or anti-mouse horseradish peroxidase-linked secondary Ab in TBTT and 5% fat-free dry milk. Chemiluminescent substrates were used to reveal positive bands visualized after exposure for 1–2 min to Hyperfilm ECL. In some samples, a shorter incubation period was employed to evaluate a large increase in COX-2 expression, resulting in apparent heterogeneity in density between figures. Protein bands were quantified using an LKB Ultrascan XL laser densitometer (Pharmacia). Immunodetection was linear between 15 and 75 μg of COX-1 and -2.

Measurement of PG production

PGs were determined in the supernatants of the cell cultures using previously validated RIA (40).

Preparation of thymocytes and coculture experiments

Thymocytes were isolated for coculture experiments from thymi of 4- to 5-wk-old mice by mechanical dissociation of the whole organ in culture medium. Thymocytes were then washed twice with HBSS, resuspended in a serum-free culture medium, and counted with trypan blue. Their viability was always >95%. Thymocytes were stained for CD4 and CD8 and analyzed by flow cytometry to assess the distribution of the different subsets.

Stromal cells were cultured to confluence on 12-well culture plates incubated overnight with indomethacin, L-759,700, NS-398, (0.1 μM), and increasing concentrations of PGE_2, or vehicle. They were then washed twice with PBS, and 1×10^6 thymocytes were added to each well in 1 ml of serum-free culture medium and incubated for 3 h at 37°C under 5% CO_2. Similarly, single cultures of thymocytes or stromal cells were incubated with the same inhibitors for the same length of time for use as controls. After 3 h of incubation, the nonadherent thymocytes were harvested by four sequential washes with serum-free culture medium (1 ml each wash), counted, and stained for flow cytometry analysis. Experiments were always performed in duplicate and were repeated at least four times.

Stromal cells and adherent thymocytes were harvested from the wells incubated with Versene (Life Technologies) for 10 min at 37°C. The cells were then collected and stained for CD4 and CD8 for qualitative analyses of thymocyte subsets adherent to stromal cell lines in coculture experiments. The number of thymocytes attached to the stromal cells was calculated by counting and analyzing the nonadherent cells by flow cytometry. To study the adhesion of DN cells to 427.1 and 1308.1 cell lines, we isolated thymocytes from RAG-$1^{-/-}$ mice. In these animals, T cell development is blocked at the DN stage because of a failure to rearrange the TCR-β locus (41).

Flow cytometric analysis and cell sorting

Thymocytes were stained using anti-CD4 FITC and anti-CD8 Red 613 mAbs. Briefly, thymocytes were spun down at 1000 rpm for 5 min at 4°C and resuspended in staining medium (HBSS/5% FCS/0.01% sodium azide) with diluted Abs for CD4 and CD8 (1:200). After 30 min incubation at 4°C, cells were then washed and resuspended in PBS and analyzed with a FACScan (Becton Dickinson, San Jose, CA). Ungated data (30,000 events per sample) were collected and analyzed using FACScan CellQuest software (Becton Dickinson). Before analysis of Ab staining, samples were gated on live cells based on forward and side scatter parameters. The relative percentages of various thymocyte subpopulations based on CD4 and CD8 expression were obtained using CellQuest on the dot-plots of flow cytometry analyses. The absolute numbers of each thymocyte subset were calculated by multiplying the total cell number by the percentage of each thymocyte population. Cell sorting was performed on thymocytes from embryonic or adult thymi stained for CD4 and CD8 as indicated. The purity of the sorted populations of thymocytes was always >99%.

Adherent cells were harvested with Versene as described previously. Cells were washed twice with staining medium and incubated for 30 min with the primary Ab, diluted in staining medium, at 4°C (ICAM-1, CD40, and CD44 were used at 1:50 dilution, FITC-conjugated hyaluronate was used at 1:75 dilution). After two washes, the cells were incubated for 30 min with FITC-conjugated secondary Abs diluted in staining medium (1:100). The flow cytometry analyses were performed as previously described.

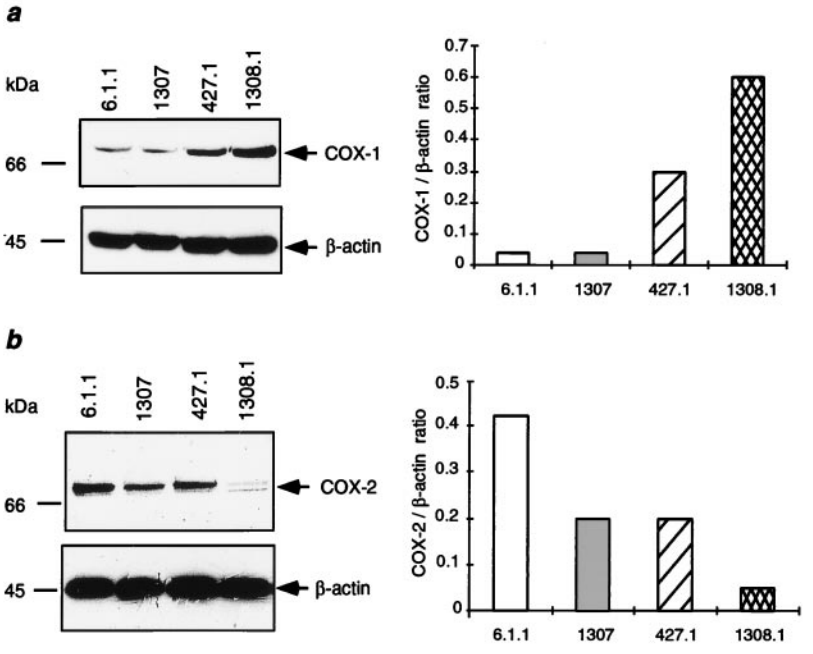

FIGURE 1. Expression of COX-1 and -2 in thymic stromal cell lines. *a*, Western blot analyses with mAbs to COX-1 and mouse β-actin in 6.1.1, 1307, 427.1.1, and 1308.1.1 thymic stromal cell lines. *b*, Western blots with mAbs to COX-2 and mouse β-actin in 6.1.1, 1307, 427.1.1, and 1308.1.1 thymic stromal cell lines. A total of 40 μg of protein were loaded in all lanes. Similar results were obtained in four independent experiments. Protein bands were quantified using a laser densitometer. The ratios between COX and β-actin densitometric values are represented in the corresponding plots.

RT-PCR analyses

Total RNA from thymocytes isolated by cell sorting was extracted with an RNAeasy minikit (Qiagen, Chatsworth, CA). First-strand cDNAs were synthesized from $\leq$1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers (Boehringer Mannheim Biochemicals, Indianapolis, IN). The mixtures were subjected to PCRs with primers specific for COX-2 (sense, 5′-CTGTACAAGCAGTGGCAA-3′, antisense, 5′-TTACAGCTCAGTTGAACGCCT-3′) spanning several intron-exon junctions. The expected DNA fragment was 530 bp. The PCR products were separated by electrophoresis through a 1.5% agarose gel containing ethidium bromide. The identity of the PCR product was confirmed by Southern blotting; the filters were hybridized using specific oligonucleotide (5′-CATTAACCCTACAGTACTAAT-3′) after 5′ end labeling with [^{32}P]ATP.

Immunocytochemistry

Cells were fixed in ice-cold 70% methanol/30% acetone, rehydrated, equilibrated in PBS buffer, pH 7.2, and stained. Briefly, specimens were incubated with 10% blocking serum diluted in PBS/1%BSA/0.02% sodium azide for 20 min. The primary Ab, either COX-1 antiserum (1:200) or COX-2 antiserum (1:400), were applied for 60 min. After washing (3 $\times$ 5 min) with PBS, the secondary FITC-labeled Ab was applied for 45 min. Sections were thoroughly washed, mounted in glycerol-gelatin, and inspected for green fluorescence under a Zeiss fluorescence microscope. Specificity of labeling was checked by appropriate controls, including single staining, omitting specific Ab, or replacing the first Ab with an irrelevant Ab. All controls gave negative results.

Statistical analysis

The results were evaluated by using ANOVA with subsequent comparisons by Student's *t* test for paired or nonpaired data, as appropriate. Statistical significance was defined as $p < 0.05$. Values are reported as means $\pm$ 1 SD. The IC_{50}s were calculated using Biosoft-Dose software (Elsevier-Biosoft, Cambridge, U.K.).

Results

Differential expression of COX-1 and -2 isozymes in thymic stromal cells

Western blot analyses were performed to evaluate constitutive COX isozyme expression in thymic stromal cell lines. COX-1 was detected in all four cell lines, although the levels of expression, normalized for β-actin, appeared to differ. The highest level of COX-1 protein expression was in the cortical cell lines 1308.1 and 427.1 (Fig. 1*a*). COX-2 protein also appeared to be constitutively expressed, although it was differentially expressed across the four cell lines (Fig. 1*b*). In this case, the highest expression was in the medullary line (6.1.1), while the lowest expression was in the cortical 1308.1 cells (Fig. 1*b*).

We explored further the expression of COX-1 and COX-2 by immunocytochemistry. Consistent with the data obtained by Western blots, the strongest reaction for COX-2 was in the 6.1.1, 1307, and 427.1 lines (Fig. 2*a*), while the reactivity of the 1308.1 line was very weak (data not shown). COX-2 immunoreactivity was particularly apparent in the perinuclear area (Fig. 2*a*), consistent with observations in other cells (42). By contrast, the 1308.1 and 427.1 lines, which were intensively positive for COX-1 (Fig. 2*b*), exhibited a diffuse granular pattern of positivity, suggesting predominant localization of this isoform in the endoplasmic reticulum (42).

COX-1 and -2 activities in thymic stromal cell lines

Expression of COX proteins in thymic stromal cell lines prompted us to investigate COX activity under basal conditions and upon stimulation with exogenous AA. We first measured the spontaneous release of PGs from each cell line over 24 h. PGE_2 was the predominant PG secreted by all the cell lines (Table I), while thromboxane (Tx) B_2 and $PGF_{2\alpha}$ only accounted for $<$10% of the total prostanoids. Additional to marked constitutive expression of COX-2, both of the medullary 6.1.1 and 1307 lines released higher amounts of PGE_2 compared with 427.1 and 1308.1 cells (Table I). Indomethacin (10 μM), a nonselective COX inhibitor, inhibited PG secretion by $>$98%.

Following incubation with 10 μM AA, PGE_2 was again the predominant PG secreted in all the cell lines (Table I). Again, the medullary cell lines (6.1.1 and 1307) generated greater amounts of PGE_2 from exogenous AA. Indomethacin (10 μM) again inhibited $>$98% the production of all the PGs from each cell line.

To address the relative contribution of each isoform to PGE_2 production, cells were treated with increasing concentrations of the selective COX-1 inhibitor, L-759,700 or of the selective COX-2 inhibitor, NS-398 (43), and stimulated with 10 μM AA. L-759,700

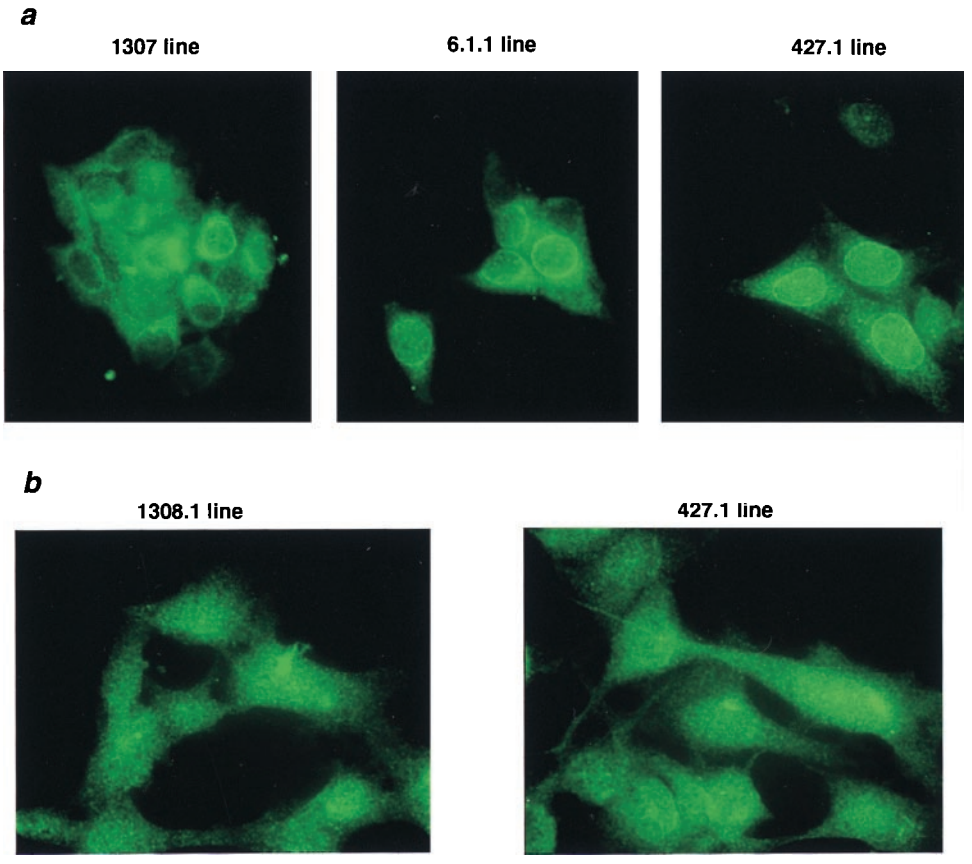

FIGURE 2. COX-1 and -2 immunocytochemistry. *a*, 1307, 6.1.1, and 427.1 cells were reacted with anti-COX-2 Ab and fluorescein-labeled secondary Ab. *b*, 1308.1 and 427.1 cells were reacted with anti-COX-1 Ab and fluorescein-labeled secondary Ab. Original magnifications, ×100.

exhibits preference for COX-1 vs COX-2 in different cellular systems (unpublished observations), while NS-398 is roughly 160-fold more potent as an inhibitor of COX-2 vs COX-1 (44). Both L-759,700 and NS-398 dose-dependently inhibited PGE_2 production, but with different potencies in each cell line. Thus, L-759,700 was more potent in inhibiting PGE_2 biosynthesis from 1308.1 cells (IC_{50} 3 nM), which predominantly express COX-1, than in the 6.1.1 and 1307 lines (IC_{50}s 185 nM and 146 nM, respectively), which predominantly express COX-2 (Fig. 3*b*). In contrast, NS-398 was more potent in inhibiting PGE_2 secretion from the cells (6.1.1 and 1307) that predominantly express COX-2 (Fig. 3*a*). 427.1 cells, which expressed both isozymes, are inhibited with intermediate potency by the selective inhibitors (Fig. 3, *a* and *b*).

In summary, the PGE_2 biosynthesis from exogenous AA appears to be derived largely from COX-1 in the cortical stromal epithelial cell line (1308.1), while it appears to come mainly from COX-2 in both medullary stromal cell lines (1307 and 6.1.1). In addition, both isoforms seem to contribute to a similar degree in PGE_2 secretion in 427.1 cells.

Differential regulation of COX expression and activity by IFN-γ

IFN-γ is an immunoregulatory lymphokine that is primarily produced by T cells and affects T cell differentiation and maturation (45). Therefore, in the thymus, stromal cells are physiologically exposed to this cytokine. Indeed 1307, 6.1.1, 1308.1, and 427.1 cell lines are IFN-γ responsive (35). For example, we have found that IFN-γ (1000 IU/ml, for 24 h) up-regulates the expression of ICAM-1 and CD44 in 1307 and 6.1.1 cell lines (data not shown), while others have reported the up-regulation of many other different molecules, including MHC class II Ags, in all these thymic stromal cell lines (35). In the present experiments, each cell line was treated with different concentrations of IFN-γ, and the levels of protein expression for each COX isozyme were investigated. We also determined whether changes in COX protein levels caused by IFN-γ were associated with changes in COX activity, measured as PGE_2 production after addition of 10 μM AA.

IFN-γ up-regulated COX-2 in both 6.1.1 and 1307 medullary stromal cell lines. COX-2 up-regulation was time-dependent,

Table I. *PGE_2 production from thymic stromal cell lines*[a]

	Cell Lines			
	6.1.1	1307	427.1	1308.1
PGE_2 (ng/10^6 cells)	3.8 ± 0.08 (n = 3)	1.4 ± 0.05 (n = 3)	0.20 ± 0.02 (n = 3)	0.70 ± 0.07 (n = 3)
PGE_2 (ng/10^6 cells) + AA (10 μM)	15.7 ± 6 (n = 8)	12.7 ± 3.5 (n = 6)	4 ± 2 (n = 8)	2 ± 1 (n = 6)

[a] Values are expressed as means ± SD.

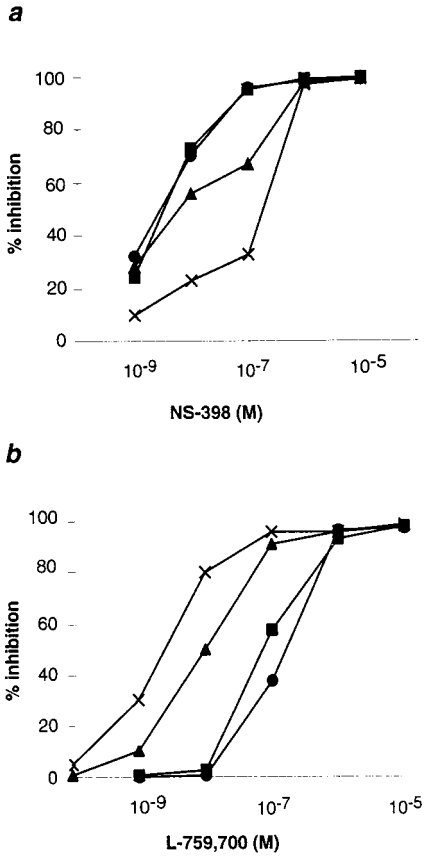

FIGURE 3. Inhibition of PGE_2 production by the selective COX-1 and -2 inhibitors. *a*, 1308.1 (×), 427.1 (▲), 1307 (■), and 6.1.1 (●) cell lines were incubated with the selective COX-2 inhibitor, NS-398, before AA incubations. PGE_2 production was measured, and the percent of inhibition of PGE_2 production (expressed in ng/10^6 cells) is shown in the plot. Results are means of two to four determinations for each concentration. *b*, The same experiments were performed in the presence of the selective COX-1 inhibitor, L-759,700.

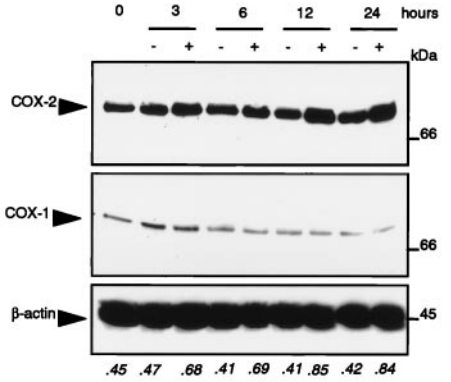

FIGURE 4. Time-dependent expression of COX-2 and COX-1 in 6.1.1 medullary stromal cell line stimulated with IFN-γ. Western blot analyses of lysates from 6.1.1 cells stimulated with 500 IU/ml of IFN-γ over 24 h. Cells were collected at different time points, as indicated. A total of 40 μg of protein were loaded on each lane. Immunodetection of COX-2 was made using specific mAbs (*upper panel*). The same membrane used for the COX-2 immunoblot was stripped, and COX-1 and β-actin immunodetection was done with a specific mAb (*middle* and *lower panels*, respectively). The numbers indicate the COX-2/β-actin ratios of the densitometric values of the bands. +, 500 IU/ml IFN-γ; −, controls.

starting 3 h after addition of the cytokine and reaching maximal expression between 12 and 24 h later (Fig. 4). No changes were observed in the levels of COX-1 protein expression under the same conditions (Fig. 4). COX-2 up-regulation was also concentration-dependent (Fig. 5, *a* and *b*) and was more prominent in the 6.1.1 compared with 1307 line (Fig. 5, *a* and *b*). PGE_2 production was also dose-dependently increased by IFN-γ (Fig. 5, *a* and *b*). Consistent with COX-2 protein expression, the increase in PGE_2 production was more pronounced in the 6.1.1 line than in the 1307 line (Fig. 5, *a* and *b*).

In contrast, IFN-γ slightly down-regulated COX-2 (Fig. 5, *c* and *d*), while it did not modify COX-1 protein expression (Fig. 6) in the 427.1 and 1308.1 cortical stromal cell lines. Interestingly, we observed a significant reduction in PGE_2 production (Fig. 5, *c* and *d*) that was greater than expected based on COX protein expression. In fact, inhibitor studies (Fig. 3) suggest that COX-2 isoform contributes roughly 50% and 30% to the PGE_2 production in the 427.1 and 1308.1 cells, respectively, while the reduction of PGE_2 production was >50% in both cell lines. To rule out an effect of IFN-γ on specific PG synthases, we measured also Tx production (as TxB_2) under the same experimental conditions. Inhibition of Tx was similar to that of PGE_2 (data not shown). This indicated that IFN-γ acts indeed at the level of COX activity rather than at the level of specific PG synthases.

In summary, IFN-γ up-regulated COX-2 protein expression and activity in the 1307 and 6.1.1 medullary cell lines, although to variable degrees. This was not observed in the cortical cell lines, where IFN-γ appeared to reduce COX-2 expression.

Impairment of adhesion of thymocyte to thymic stromal cells by COX-2 inhibitors

The interaction of thymocytes with stromal cells is believed to be necessary for development of the T cell lineage (46). Thymocytes, freshly isolated from young adult (4–5 wk) mouse thymus, were cocultured with each of the four stromal cell lines. Preliminary experiments were performed to identify qualitatively the thymocyte subsets that adhered to each stromal cell line. More than 80% of the thymocytes adherent to the cortical 427.1 cells were phenotipically DNs, while ~80% of thymocytes adherent to the cortical 1308.1 line were DPs and the remaining 20% were DNs. The vast majority of thymocytes adherent to the medullary 1307 and 6.1.1 lines (~80%) were DPs. In addition, CD4 SP thymocytes bind to 6.1.1 cells. The differential capacity of these stromal cell lines to bind different thymocyte subsets is consistent with their origins. Both 427.1 and 1308.1 lines derive from the cortex, where the majority of resident thymocytes are DN or DPs, while medullary cells, such as 6.1.1 and 1307, would be expected to reflect interactions with DPs (at the corticomedullary junction) and CD4 or CD8 SP lymphocytes, which reside in the medulla.

Next, we studied whether COX-1 or -2 modulated the adhesiveness of thymocyte subpopulations to stromal cells. Neither indomethacin, NS-398, nor L-759,700 influenced the adhesion of DN thymocytes to the 1308.1 and 427.1 cortical cell lines (data not shown). The adherence of DP cells to 1308.1 was also unaffected by the COX inhibitors (data not shown).

In contrast to our observations with the cortical stromal cell lines, NS-398 dose-dependently inhibited the adhesion of DP cells to the medullary 1307 and 6.1.1 cells (Fig. 7). The selective COX-2 inhibitor, NS-398, also impaired the adhesion of CD4 SP cells to 6.1.1 cells (Fig. 7). Indomethacin had the same effect (data not shown), while the selective COX-1 inhibitor L759,700 at doses

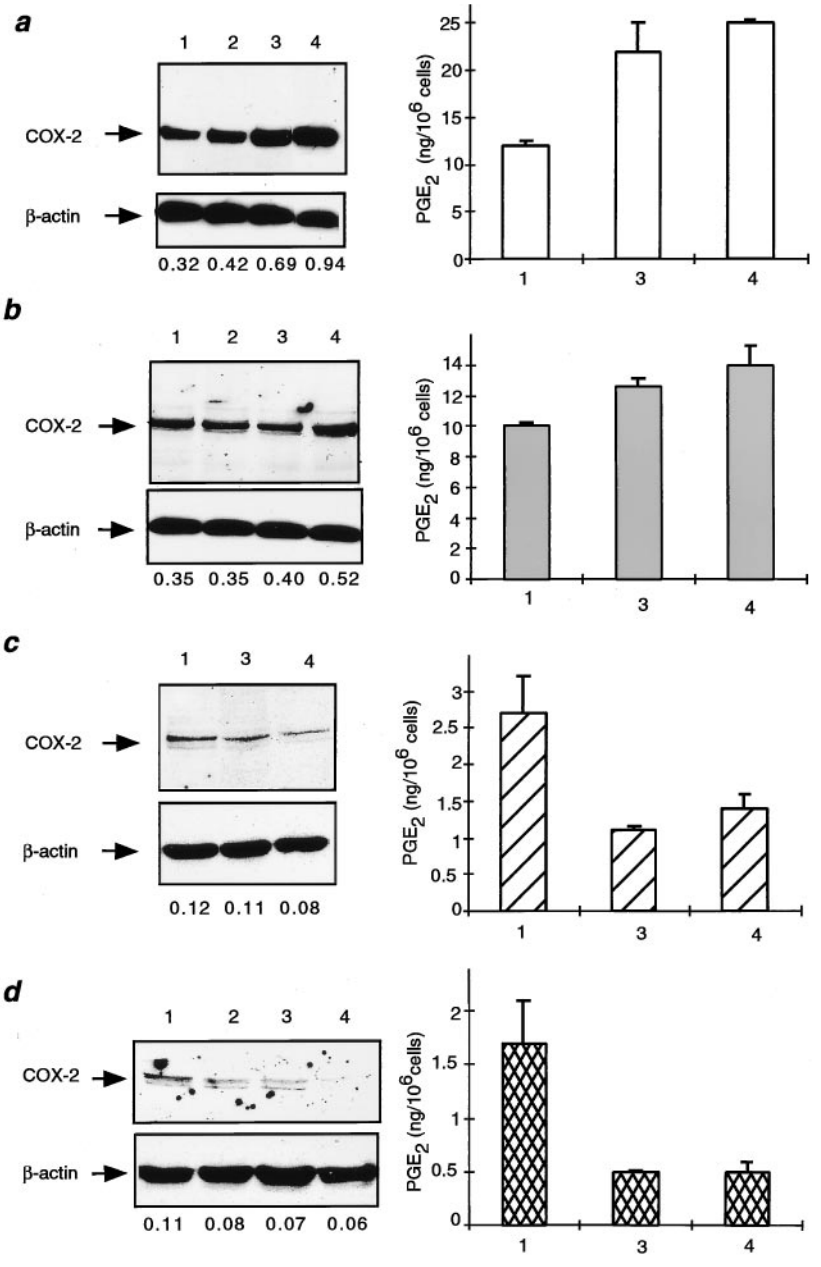

FIGURE 5. Dose-dependent effect of IFN-γ on COX-2 protein expression and PGE_2 production in thymic stromal cell lines. Western blot analyses of lysates from cells stimulated with different doses of IFN-γ over 20 h. Immunodetection of COX-2 using mAbs against COX-2 (*left panels*). The same membrane used for the COX-2 immunoblot was stripped, and β-actin immunodetection was done with a specific mAb (as shown) as control. *a*, 6.1.1 cell line (40 μg of protein); *b*, 1307 cell line (40 μg of protein); *c*, 427.1. cell line (40 μg of protein); *d*, 1308.1. cell line (50 μg of protein). *Lane 1*, controls; *lane 2*, 50 IU/ml IFN-γ; *lane 3*, 500 IU/ml IFN-γ; *lane 4*, 1000 IU/ml IFN-γ. The numbers indicate the COX-2/β-actin ratios of the densitometric values of the bands. In different experimental sets, cells incubated with different concentrations of IFN-γ for 20 h were washed and treated with 10 μM AA. PGE_2 production was measured and is shown (*right panels*).

up to 0.1 μM was ineffective (data not shown). Inhibition of adherence by NS-398 was dose-dependently rescued by adding exogenous PGE_2 to the cocultures (Fig. 7). NS-398 and indomethacin, under these experimental conditions, did not affect the counts of the adherent stromal cells and did not have any effect on either the counts or the phenotypic distribution of the thymocytes alone. Therefore, the reduction of adherent thymocytes cannot be attributed to a possible reduction in the number of stromal cells. Moreover, expression of COX-2 m-RNA in thymocyte subsets (DN, DP, 4SP) was excluded by RT-PCR (data not shown). Thus, the effects of NS-398 are attributable to inhibition of COX-2 in stromal cells (Fig. 1*b*).

Finally, we investigated whether NS-398 or indomethacin modulated the expression of adhesion molecules present on the medullary 1307 and 6.1.1 cells. We could not find any effect of COX inhibitors (NS-398 up to 1 μM and indomethacin up to 10 μM) on levels of expression of ICAM-1, CD40, CD44, hyaluronate receptors (detected by flow cytometry), or E-cadherin (detected by Western blot analyses).

Discussion

Interactions between the thymocytes and the thymic stroma are known to be critical to T cell development (33, 47). Thymic stromal cells appear to provide signals to developing thymocytes through a combination of cell-cell contact and soluble factors. For example, interactions involving the T cell receptor on thymocytes and MHC molecules on the stromal cells are known to regulate the selection of the TCR repertoire (46). Furthermore, TCR-mediated interactions alone seem insufficient to promote either positive or negative selection and it is likely that unknown accessory signals from the stromal cells are also required (33). Factors released from stromal cells, such as IL-7 and stem cell factor, as well as constituents of the extracellular matrix, appear to modulate colonization, proliferation, maturation, and migration of thymocytes in the thymus (33, 45, 47). Elucidation of the signals emanating from the heterogenous stromal cell types is of fundamental importance if the mechanisms that regulate T cell formation and function are to be understood.

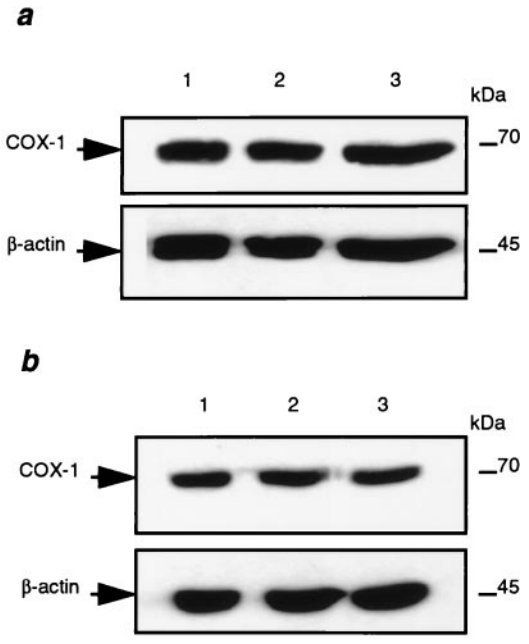

FIGURE 6. Effect of different doses of IFN-γ on COX-1 protein expression in cortical thymic stromal cell lines. Western blot analyses of lysates from 1308.1 and 427.1 cell lines stimulated with different doses of IFN-γ over 20 h. Immunodetection of COX-1 was performed using mAbs against COX-1. The same membrane used for the COX-1 immunoblot was stripped, and β-actin immunodetection was done with a specific mAb (as shown) as control. *a*, 1308.1 cell line (40 μg of protein); *b*, 427.1. cell line (40 μg of protein). *Lane 1*, controls; *lane 2*, 500 IU/ml IFN-γ; *lane 3*, 1000 IU/ml IFN-γ.

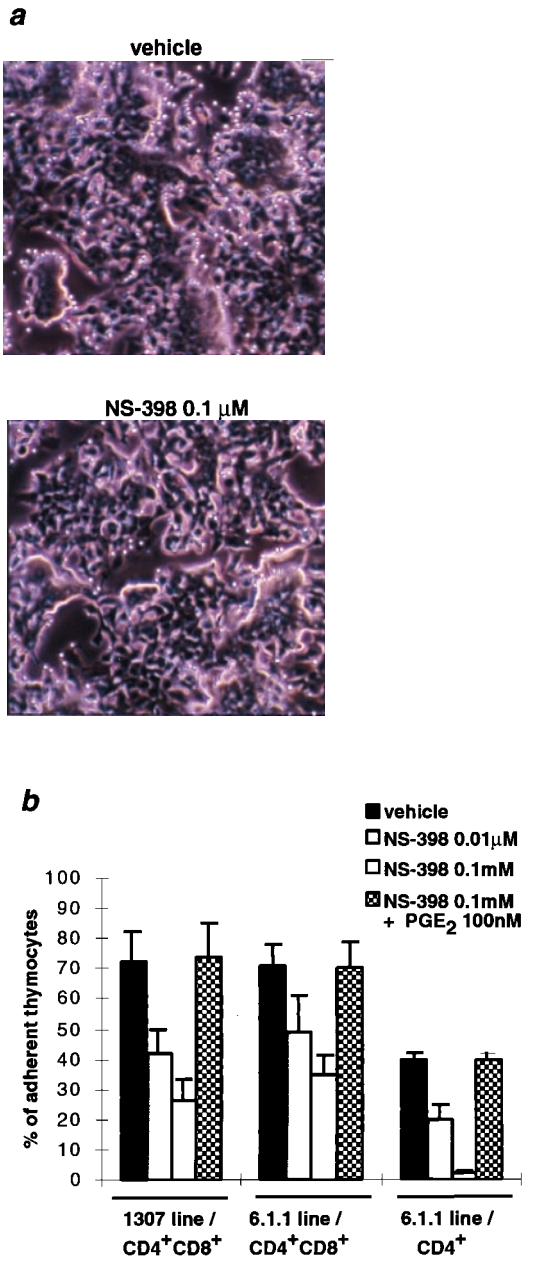

FIGURE 7. Effect of COX-2 inhibition on attachment of thymocytes to stromal cells. *a*, Confluent 6.1.1 cells were used for thymocyte adhesion assay. Adhesion was evident to the stromal cells (*left panel*), but treatment of stromal cells with the COX-2 inhibitor, NS-398, significantly decreased the basal adhesion (*right panel*). Original magnification, ×20. *b*, Quantification of thymocyte adhesion to stromal cells. Thymocytes adherent to stromal cells, expressed as percent of input thymocytes, are represented in the diagram. Values are means ± SD ($n = 3$) of one representative experiment.

Our previous studies have shown the presence of both COX isozymes in the embryonic murine thymus. Both MHC class II-positive stromal cells in embryonic thymus and medullary stroma in adult thymus stain positively for COX-2 (32), raising the possibility that PG formation might represent a signaling mechanism(s) between stroma and developing thymocytes.

The thymic stroma is a complex network of phenotypically distinct cells, such as cortical and medullary epithelial cells, nurse cells, nonepithelial dendritic cells, macrophages, and fibroblasts (34). This heterogeneity seems likely to reflect functional specialization (33, 34). For example, cortical epithelial cells appear to support positive selection of DP thymocytes, and the medullary stroma is involved in further nurturing, maturation, induction of self-tolerance, and circulation of CD4 and CD8 SP lymphocytes (33, 34).

Given these observations, we investigated the expression, activity, regulation, and function of COX isozymes in four different thymic stromal cell lines that are representative of thymic subcapsular cortex nurse cells (427.1), cortical reticular cells (1308.1), medullary interdigitating-like cells (6.1.1), and medullary epithelial cells (1307) (35).

COX-2 appears to be the predominant COX isoform, in terms of protein expression and activity in both the medullary thymic stromal cell lines 6.1.1 and 1307. Selective COX inhibitors were used to demonstrate that production of PGE_2, the predominant PG formed, derives largely from COX-2 in these medullary stromal cells. These findings are consistent with our observation that COX-2 is the predominant isozyme in the medullary stroma of mouse adult thymus by immunohistochemistry (32). Both the expression and activity of COX-2 appear to be regulated in the medullary cell lines. Thus, IFN-γ dose-dependently increased COX-2 protein expression and PGE_2 production in the 6.1.1 and 1307 cells, albeit with different potencies (Fig. 5). Furthermore, pharmacological inhibition of COX-2 impaired the adhesion of DP and CD4 SP thymocytes to these medullary stromal cell lines (Fig. 7). Addition of exogenous PGE_2 to the cocultures rescued this effect of the selective COX-2 inhibitors. Interestingly, transgenic mice that overexpress IFN-γ exhibit an increase in the number of CD4 and CD8 SP lymphocytes in their thymi. This is associated with a reduction in circulating lymphocytes, reflective of a disruption in the normal processes that govern release of mature lymphocytes into the bloodstream (48). In the present study, IFN-γ up-regulated

COX-2 in medullary cells and COX-2-dependent PGE_2 production enhanced the adhesion of CD4 SP lymphocytes to them.

COX-2-dependent PG formation has previously been implicated in the regulation of adhesive processes involving nonthymic cells. For example, COX-2 overexpression increases the adhesion of epithelial cells to extracellular matrix molecules (49). It has also been reported that COX-2 induction (50) or overexpression are associated with up-regulation of adhesion molecules in epithelial cells (49). Because of these observations, we examined the effects of selective COX-2 inhibition on expression of some adhesion molecules and extracellular matrix receptors by the thymic stromal medullary 6.1.1 and 1307 cell lines. Despite the role of COX-2-dependent PGE_2 formation in modulating the adhesive interactions of these cells with thymocytes, we failed to detect any variation in the stromal expression of ICAM-1, E-cadherin, CD40, CD44, or hyaluronate binding proteins in the presence of the selective COX-2 inhibitor, NS-398, or of the nonselective inhibitor, indomethacin. Nevertheless, we and others have found that various subtypes of PGE_2 receptors are expressed on isolated thymocytes at different stages of their development (Ref. 23 and our unpublished observations). Therefore, it is possible that COX-2-dependent PGE_2 production from stromal cells acts on neighboring thymocytes to enhance their adhesiveness. It has been already shown that PGE_2 and cAMP both increase the binding of immature DN and DP thymocytes to extracellular matrix proteins (51). Similar mechanism(s) may be involved in the adhesion of thymocytes to medullary stromal cells. It is also possible that PGE_2 modulates the stromal synthesis of soluble chemoattractant factors that are known to be involved in the interaction of thymocytes with stromal cells (33). Therefore, it will be important to determine whether the spectrum of chemokines produced by stromal cells is affected by COX inhibitors. We have recently shown that COX-2-dependent PGE_2 formation acting via the EP1 subtype appears important in the positive selection of the CD4 SP lymphocytes (32). Whether the role of PGE_2 in modulating thymocyte interactions with medullary stromal cells is relevant to this process remains to be determined.

Expression and activity of the COX isozymes was markedly different in the two cell lines that originated from the cortical stroma (1308.1 and 427.1 lines). Both expression and activity of COX-1 was more prominent than COX-2 in these cells (Figs. 1 and 3). Furthermore, IFN-γ caused a slight, dose-dependent down-regulation of COX-2 protein expression, with a marked (>50%) reduction in COX activity in the cortical stromal cell lines, while COX-1 protein expression was unchanged (Figs. 4–6). Selective inhibition of COX-1 suggests that it accounts for roughly 70% and 50% of the total COX activity in the 1308.1 and 427.1 cell lines, respectively. These observations raise the possibility that IFN-γ down-regulates COX-1 activity without altering its expression in cortical cells. A discrepancy between the effects of cytokines on COX protein expression and activity has been previously reported in other cells. For example, TNF-α increased AA-induced PGE_2 formation by human airway smooth muscle cells without inducing expression of either COX-1 or -2 proteins (52). Similarly, nitric oxide donors suppress COX activity, despite increasing COX-2 protein expression in human endothelial cells (53). Again, selective inhibitors of either COX isozyme failed to influence the attachment of DN or DP thymocytes to the cortical cell lines, in contrast to our observations with medullary stromal cells. Thus, the differential regulation of the COX isozymes in cortical vs medullary cells suggests that PG formation may serve to differentiate functionally these two components in the thymic stroma.

We measured PG synthesis in stromal cells in the absence or presence of exogenous AA. We found no differences in the profile or the relative amounts ($PGE_2 > PGF_{2\alpha} > TxA_2$) of PG synthesized under the two experimental conditions in the four cell lines. Furthermore, selective COX-1 or COX-2 inhibitors decreased PGE_2 production from exogenous or endogenous AA with potencies that reflected the relative predominance of isoform expression. Thus, the isozyme available, rather than the source of the substrate, seems to determine which isozyme catalyzes PG formation in stromal cells. Similar observations have been made in human endothelial cells (54).

PGE_2 is the most abundant PG synthesized by both medullary and cortical thymic stromal cell lines. Lesser amounts of $PGF_{2\alpha}$ and TxB_2 are synthesized by thymic stromal cell lines. Indeed, both Tx synthase and thromboxane receptors are present in human and murine thymus and thymocytes (21, 23, 24). Mouse thymi depleted of hemopoietic cells with 2′-deoxyguanosine produce PGE_2, $PGF_{2\alpha}$, and TxB_2, presumably from the residual thymic epithelium (29).

These observations prompt interest in how deletion of genes encoding COX isozymes or EPs might affect central (thymus) and/or peripheral immune function. Although little information is presently available, fetal thymocyte maturation is altered in COX isozyme deficiency, and adult COX-2-deficient mice are susceptible to spontaneous peritonitis (32, 55). Furthermore, there are reports that isozyme nonspecific COX inhibitors interfere with the induction of thymic tolerance in vivo in some rodent models (56, 57).

In summary, we report that COX-1 expression and activity appears to predominate in cells that originate from cortical thymic stroma. By contrast, COX-2 predominates in thymic medullary stromal cell lines. The lymphokine, IFN-γ, differentially modulates the expression and activity of COX-2, rather than COX-1, in medullary stromal cell lines, and COX-2-dependent PGE_2 modulates adhesion of thymocytes to those cells. PG formation by stromal cells may contribute to the compartmentalized regulation of T cell development and function in the thymus.

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