**Brief Definitive Report**

**Thromboxane A₂ Receptor Is Highly Expressed in Mouse Immature Thymocytes and Mediates DNA Fragmentation and Apoptosis**

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**Summary**

We have recently revealed that the thymus is the organ showing the highest expression of thromboxane (TX) A₂ receptor in mice. In this study, thymic cell populations expressing the receptor were identified, and the effects of a TXA₂ agonist on these cells were examined. Radioligand binding using a TXA₂ receptor-specific radioligand revealed a single class of binding sites in the thymocytes with an affinity and specificity identical to those reported for the TXA₂ receptor. The receptor density in these cells was comparable to that seen in blood platelets. This receptor is most highly expressed in CD4⁻8⁻ and CD4⁺8⁺ immature thymocytes, followed by CD4⁺8⁻ and CD4⁺8⁺ cells. The receptor density in splenic T cells was less than one fifth of that in CD4⁺8⁺ cells and no binding activity was detectable in splenic B cells. The addition of a TXA₂ agonist, STA₂, to thymocytes induced the disappearance of the CD4⁺8⁺ cells in a time- and concentration-dependent manner and caused DNA fragmentation. These changes were blocked by a specific TXA₂ antagonist, S-145. These results demonstrate that TXA₂ induces apoptotic cell death in immature thymocytes by acting on the TXA₂ receptor on their cell surface and suggest a role for the TXA₂/TXA₂ receptor system in the thymic micro-environment.

Prostanoids such as prostaglandins (PGs), thromboxane (TX), and leukotrienes (LTs) are a family of oxygenated arachidonate metabolites that exert a variety of actions to maintain local homeostasis in the body (1, 2). Their actions as inflammatory mediators are well known; for example, PGE₂ causes vasodilatation to increase local blood flow, LTβ₄ induces chemotaxis and activates neutrophils, and LTC₄ increases vascular permeability. Some of these compounds are produced upon immunologic challenge and are mediators of anaphylaxis. A recent report also suggests the participation of PGE₂ to the apoptotic cell death of ovarian surface epithelial cells (3). However, little is known about their action in the thymus. TXA₂ is a very unstable arachidonate metabolite with a half life of about 30 s under physiological conditions. Yet, it is a potent stimulator of platelets and a constrictor of vascular and respiratory smooth muscles, and is presumed to play a role in thrombosis, myocardial infarction, and bronchial asthma (4). This compound is synthesized by a specific enzyme, TX synthase, and functions through a cell surface receptor. We have cloned cDNAs for the human and mouse TXA₂ receptors and revealed that they belong to the family of rhodopsin-type receptors (5, 6). Northern blot analysis in various mouse organs showed that the TXA₂ receptor mRNA is most abundantly expressed in the thymus, followed by the spleen and lung (6). On the other hand, TX synthase is richest in platelets, followed by macrophages (7). In the thymus, it is present in the cortical epithelial cells and dendritic cells of the medulla (8, 9). These findings urged us to investigate the localization of the TXA₂ receptor and its possible role in the thymus. In this study, using a radioligand binding assay, we determined the distribution of the TXA₂ receptor in cells of the murine thymus and spleen, and found that this receptor is predominantly expressed in immature CD4⁻8⁻ and CD4⁺8⁺ thymocytes. We further found that a TXA₂ agonist evokes DNA fragmentation and apoptosis in these cells.

**Materials and Methods**

**Materials.** 5Z-7-(3-endo-phenylsulphonylamino-bicyclo[2.2.1] hept-2-exo-yl) heptenoic acid (S-145) and [³H]S-145 were gifts from Shionogi Research Laboratories (Osaka, Japan). 9,11-epithio-11,12-methano-thromboxone A₂ (STA₂), 9,11-dimethylmethano-11,12-thromboxone A₂ (ST₄), and 9,11-dimethylmethano-11,12-thromboxone A₂ (ST₅).
methyl-13,14-dihydro-13-aza-14-oxo-15-cyclopentyl-16,17,18,19, 20-pentanor-15-epi-thromboxane A2 (ONO3708), PGE2, PGF2α, PGE3, and TXB2 were gifts from Ono Pharmaceuticals (Osaka, Japan). 15(S)-hydroxy-11,9-epoxymethano-prosta-5Z,13E-dienoic acid (U46619) and iloprost were obtained from the Upjohn Company (Kalamazoo, MI) and Amersham International (Amersham International, England), respectively. Phycocerythrin-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD8 mAbs were purchased from Becton Dickinson & Co. (Mountain View, CA). Diphenylamine was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade.

**Cell Preparation and Culture.** BALB/c mice of 5–7 wk-of-age were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used after at least 1 wk of maintenance in our laboratory. Thymocyte suspensions were prepared in Eagle’s MEM by mincing the thymus and filtering dissociated cells through a nylon mesh (pore size, 40 μm) (10). To examine the distribution of the TXA2 receptor, the cells were initially treated with either anti-CD4 or anti-CD8 Ab or both followed by treatment with rabbit complement, and separated into fractions of CD4+ and CD4- cells using a nylon wool column and a Sephadex G-10 column. Splenic B cells were obtained by passing dissociated cells successively through a nylon wool column and a Sephadex G-10 column. Splenic B cells were obtained as reported previously (11). The purity of the splenic cell fractions was in the range of 95–99%. In some experiments, splenic CD4+ cells were sorted using a cell sorter (ABCAS-100; Showa Denko Co., Ltd., Tokyo, Japan).

**Binding Assay.** Cells, washed once with phosphate buffer (10 mM sodium phosphate, pH 7.4, containing 10 mM EDTA, 5 mM KCl, and 135 mM NaCl), were suspended in Hepes-saline (20 mM Hepes, 5 mM KCl, 5 mM MgCl2, and 140 mM NaCl, pH 7.4) and the binding assay was carried out as reported previously (12). Cells were incubated with various concentrations (0.5–8 nM) of [3H]S-145 for Scatchard analysis, or with 5 nM [3H]S-145 and various concentrations of TXA2 analogs and PGs in the displacement experiments. Nonspecific binding was determined as the binding in the presence of 20 μM unlabeled S-145.

**DNA Fragmentation.** Thymocytes were washed once with Eagle’s MEM and suspended at 1.8–5.3 x 10⁶ cells/ml in RPMI 1640 containing 1% BSA. The cells were then incubated at 37°C in a humidified atmosphere of 5% CO2 in the presence of relevant agents for 20 h. DNA fragmentation was measured as described by Wyllie (13). Briefly, the cells were lysed with 0.5% Triton X-100, and centrifuged at 27,000 g to separate fragmented DNA in the supernatant from chromatin in the pellet. DNA in the supernatant and pellet was then precipitated by the addition of trichloroacetic acid and quantified using the diphenylamine reaction (14). The percent fragmentation indicates the ratio of DNA in the supernatant to the total DNA recovered in the supernatant and pellet. The values are presented after subtracting DNA fragmentation in the control experiment, which was 10–15% on average.

**Flow Cytometry.** Thymocytes were incubated at 37°C in RPMI 1640 containing 10% FCS with or without relevant agents for the indicated periods. The cells were stained with phycoerythrin-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD8 mAbs. Approximately 5 x 10⁶ cells were analyzed by a FACScan* two-color flow cytometer (Becton Dickinson & Co.) as reported previously (10, 11).

**Results and Discussion**

The TXA2 Receptor Is Highly Expressed in Immature Thymocytes. A thymocyte suspension was prepared from 5–7-wk-old BALB/c mice and used in the binding assay for the TXA2 receptor employing [3H]S-145 as a radioligand (12, 15). Scatchard analysis of the results showed a single class of binding sites with KD and Bmax values of 2.03 ± 0.15 nM and 788 ± 15 fmol/10⁶ cells (~4,800 sites/cell), respectively (Mean ± SEM, n = 4) (Fig. 1a). This [3H]S-145 binding was effectively displaced by TXA2 antagonists and agonists with a rank order of potency of S-145>ONO3708>STA2>U46619. The binding was partially displaced by a PGF2α agonist, iloprost, and PGE2, while TXB2, PGE3, and PGE5α were ineffective at concentrations up to 10 μM (Fig. 1b). The affinity and specificity of binding were identical to respective values reported for the TXA2 receptor in other cells (12, 15–17). These results indicated that this binding activity represents the TXA2 receptor, and that the thymocytes express this receptor at a density comparable to that found in blood platelets, ~2,000 sites/cell (18, 19). By incubation in buffer alone or with anti-CD4 Ab, anti-CD8 Ab, or both Abs, the thymocytes were fractionated into four fractions according to their expression of CD4 and CD8 (11). A cell suspension was also prepared from mouse spleen cells and

![Figure 1](https://example.com/figure1.jpg)
separated into T and B cell fractions as described previously (11). These cell fractions were then used in the binding assay and the density of the TXA₂ receptor in each lymphocyte population was calculated. As shown in Table 1, CD₄⁻⁻ and CD₄⁺⁺ T lymphocytes expressed the highest level of receptor, which was about twice that of CD₄⁻⁺ and CD₄⁻⁺ cells and more than five times that of splenic T cells. The TXA₂ receptor was not detected in B cells with the method used. These results clearly show that the TXA₂ receptor is expressed early in development of T cells in the thymus and its expression decreases gradually during maturation. It is not clear from these results, however, whether the receptor is expressed in all CD₄⁻⁻ and CD₄⁺⁺ T cells or localized to some subpopulation(s) of these cells.

A TXA₂ Agonist, STA₂, Induces DNA Fragmentation in Thymocytes and a TXA₂ Antagonist, S-145, Inhibits this Action. The preceding results suggest that TXA₂ can act on thymocytes and induce some biological response. TXA₂ is known to evoke phosphatidyl inositol breakdown through a GTP binding protein and to induce a rise in the intracellular Ca²⁺ concentration and activate protein kinase C (20, 21). Because several reports (13, 22–24) implicated a rise in Ca²⁺ concentration and/or protein kinase C activation in DNA fragmentation and apoptosis of immature thymocytes, we added a TXA₂ agonist, STA₂ (18, 25), to thymocytes and examined its effects. As shown in Fig. 2 a, STA₂ induced DNA fragmentation of thymocytes in a concentration-dependent manner in 20 h of incubation. This effect was half maximal at 20.7 μM STA₂ and reached a plateau at 10 μM, which was about 15% of the total DNA was fragmented. Under this condition, the cell viability, examined by the trypan blue dye exclusion method, decreased to 81.6 ± 1.3% compared with 98.5 ± 1.1% in the control (mean ± SEM, n = 13). This dose-dependency correlated well with that observed for platelet aggregation to STA₂ in platelet rich plasma (18). The STA₂-induced DNA fragmentation was inhibited in a concentration-dependent manner by a specific TXA₂ antagonist, S-145 (26) (Fig. 2 b), suggesting that it was a receptor-mediated process. Actinomycin D, 2 μg/ml, completely inhibited the STA₂-induced increase in DNA fragmentation, suggesting the involvement of protein synthesis in this process as reported for steroid-induced DNA fragmentation (27). An inactive metabolite of TXA₂, TXB₂, did not induce DNA fragmentation. Some other prostaglandins, PGD₂ and PGF₂α at 3 μM concentration had no effect, whereas PGE₂ induced DNA fragmentation comparable to that seen with STA₂; 16% fragmentation at 3 μM. Because the affinity of PGE₂ for the TXA₂ receptor was very low (Fig. 1 b), it probably acted on other types of PG receptors. We have recently cloned cDNAs for the EP₂ and EP₃ subtypes of the PGE₂ receptor, and found significant expression of their mRNAs in the mouse thymus (28, 29).

### Table 1. Binding of [³H]S-145 to Lymphocyte Populations of Mouse Thymus and Spleen

<table>
<thead>
<tr>
<th>Organ</th>
<th>Population</th>
<th>[³H]S-145 Binding (fmol/10⁶ cells)</th>
</tr>
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<tbody>
<tr>
<td>Thymus*</td>
<td>Whole T cells</td>
<td>788 ± 15</td>
</tr>
<tr>
<td></td>
<td>CD₄⁻⁻ cells</td>
<td>934 ± 167</td>
</tr>
<tr>
<td></td>
<td>CD₄⁺⁺ cells</td>
<td>1178 ± 74</td>
</tr>
<tr>
<td></td>
<td>CD₄⁻⁺ cells</td>
<td>481 ± 52</td>
</tr>
<tr>
<td></td>
<td>CD₄⁺⁺ cells</td>
<td>404 ± 38</td>
</tr>
<tr>
<td>Spleen</td>
<td>T cells</td>
<td>174 ± 15</td>
</tr>
<tr>
<td></td>
<td>B cells</td>
<td>&lt;60</td>
</tr>
</tbody>
</table>

* Lymphocytes were prepared from mouse thymus and separated into fractions of CD₄⁻⁻ plus CD₄⁺⁺ cells, CD₄⁺⁺ plus CD₄⁻⁻ cells, and CD₄⁻⁻ cells as described in Materials and Methods. A [³H]S-145 binding study was carried out in these fractions and unfractionated cell suspensions. Proportions of the CD₄⁻⁻, CD₄⁺⁺, CD₄⁻⁻, and CD₄⁻⁻ cells in these fractions were determined by flow cytometric analysis using anti-CD4 and anti-CD8 mAbs. The binding activity in each cell population was then calculated from binding values in the above fractions on the basis of their proportions. 

Mean ± SEM are shown (n = 3–4).
Contrary to the effect on thymocytes, STA2 could not induce DNA fragmentation in splenic T cells (data not shown).

STA2 Causes Apoptosis in CD4+8+ Thymocytes. The above results clearly showed that a TXA2 agonist acted on a specific receptor and induced DNA fragmentation and cell death in thymocytes. To identify the thymocyte subpopulations affected by this treatment, we carried out flow cytometric analysis using anti-CD4 and anti-CD8 antibodies. As shown in Fig. 3a, this analysis revealed that the proportion of viable CD4+8+ cells was preferentially decreased by STA2 treatment, while the proportions of CD4+8- and CD4-8+ cells increased. S-145 significantly inhibited the STA2-induced decrease in the CD4+8+ cell population (data not shown). The effects of the STA2 on the CD4+8- cells could not be determined due to their small numbers. A similar analysis was then performed on a cell population containing dead thymocytes. To identify the thymocyte subpopulations affected by this treatment, we carried out flow cytometric analysis using anti-CD4 and anti-CD8 antibodies, and analyzed by a FACScan two-color flow cytometer as described in Materials and Methods. The numbers in the figures represent the percentage of cells included in each quadrant.

Figure 3. Flow cytometric analyses of mouse thymocyte populations after treatment with a specific TXA2 agonist, STA2. (a) and (b) Whole thymocytes were incubated for 48 h, stained with fluorescence-labeled anti-CD4 and anti-CD8 antibodies, and analyzed by a FACScan two-color flow cytometer as described in Materials and Methods. The numbers in the figures represent the percentage of cells included in each quadrant. 0 h, cells before incubation. 48 h (-STA2) and 48 h (+STA2), cells treated without or with STA2 for 48 h, respectively. Only viable cells (a) or both viable and dead cells (b) were gated by forward and side-scatter. (c) Analysis on an isolated CD4+8+ cell population. CD4+8+ cells were sorted with a cell sorter and incubated with or without 10 μM STA2 for 72 h, and stained as described. Only viable cells were gated for analysis.

Cells increased. S-145 significantly inhibited the STA2-induced decrease in the CD4+8+ cell population (data not shown). The effects of the STA2 on the CD4+8- cells could not be determined due to their small numbers. A similar analysis was then performed on a cell population containing dead thymocytes. To identify the thymocyte subpopulations affected by this treatment, we carried out flow cytometric analysis using anti-CD4 and anti-CD8 antibodies, and analyzed by a FACScan two-color flow cytometer as described in Materials and Methods. The numbers in the figures represent the percentage of cells included in each quadrant. 0 h, cells before incubation. 48 h (-STA2) and 48 h (+STA2), cells treated without or with STA2 for 48 h, respectively. Only viable cells (a) or both viable and dead cells (b) were gated by forward and side-scatter. (c) Analysis on an isolated CD4+8+ cell population. CD4+8+ cells were sorted with a cell sorter and incubated with or without 10 μM STA2 for 72 h, and stained as described. Only viable cells were gated for analysis.

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The thymic microenvironment is thought to be very important for T cell development. It is presumed to provide cell to cell interactions and soluble factors to pursue matura-
situations. Though a TXA2 agonist induced apoptosis in isolated thymocyte suspension, TXA2 may behave as a growth signal rather than as an apoptotic signal in the physiological context. Growth stimulatory activity of TXA2 has already been reported in aortic smooth muscle cells (17) and suggested for peripheral T cells (39). It remains to be determined in what physiological context and at what developmental stage the TXA2/TXAz receptor system acts in the thymus. TXA2 is synthesized through sequential reactions by cyclooxygenase and TX synthase, and it has been shown that TIS10, a cyclooxygenase that can be induced by cytokines and growth factors, is highly expressed in the thymus of the neonatal mouse and disappears quickly after birth (40, 41). The precise role of the TXA2/TXAz receptor system will be clarified by determining the physiological stimuli required to evoke TXA2 production in the thymus, and the present report should facilitate studies on its role for intrathymic T cell development.

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