We investigated the effect of transforming factor factor-β1 (TGF-β1) on thromboxane B2 (TXB2) and prostaglandin E2 (PGE2) production in *in vitro* silica dust-exposed rat alveolar macrophages (AM). In the presence of 5 μg of anti-TGF-β1 antibodies, TXB2 production decreased, but PGE2 production increased. Addition of 2 ng of TGF-β1 to the culture medium potentiated TXB2 production, but PGE2 production apparently did not change. At 50 ng of TGF-β1, TXB2 production decreased, and PGE2 production varied. Our data suggest that in rat AM: (1) both endogenous and exogenous TGF-β1 regulate TXB2 production; and (2) in the absence of endogenous TGF-β1, the liberation of PGE2 increases; however, exogenous TGF-β1 does not have a regulatory effect on PGE2.

**Key words:** Rat alveolar macrophages, PGE2, TGF-β1, TXB2

### Introduction

Chronic airway inflammation and lung fibrosis are common features of prolonged exposure to mineral particles of silica in humans and in rodents. Alveolar macrophages (AM) have an essential role in lung clearance and defence mechanisms against inhaled particles. They fulfill these functions via inflammatory mediators produced upon macrophage activation. Among the many mediators are arachidonic acid metabolites such as thromboxane A2 (TXA2) and prostaglandin E2 (PGE2). Thromboxane A2 (of which TXB2 is the stable metabolite) is a very potent platelet aggregating factor and pulmonary vasoconstrictor. A recent study of the regulatory effect of TXB2 on the proliferation of vascular smooth muscle cells from rats demonstrated a rapid build-up of cytoskeletal protein in these cells in hypertension, suggesting that TXB2 may play a role in the remodelling of the vascular wall in hypertension.

Prostaglandin E2 is one of the key inflammatory mediators with multiple functions. In the lung, it induces bronchodilation and causes an increase in epithelial cell chloride secretion. PGE2 induces vasodilatation in unventilated foetal lung.

Another mediator that is produced by alveolar macrophages is transforming growth factor-β1 (TGF-β1). TGF-β1 is a multifunctional peptide. TGF-β1 can influence cell proliferation or differentiation and other functions in various types of cells, some of which are related to inflammation and tissue fibrosis. For example, TGF-β1 increases the rate of fibronectin gene transcription, can down-regulate interleukin-1β receptors, and inhibits interleukin-6 release from fibroblasts. The most profound effect of TGF-β1 is its ability to enhance the formation of extracellular matrix. TGF-β1 promotes the formation of collagen and fibronectin in fibroblasts of human, rat and mouse origin. From *in vivo* studies, it has been shown that TGF-β1 can stimulate the formation of the typical granulation tissue found in tissue repair.

Although pulmonary macrophage-derived mediators have a pivotal role in lung inflammation and fibrosis, the interrelationship between these mediators is just beginning to be understood. We have shown the very early release (15 min) of TGF-β1 from silica dust-exposed rat alveolar macrophages. The amount of TGF-β1 released at this time was the greatest over 24 h kinetic studies, and preceded the production of TXB2 and PGE2. To further unravel the interrelationship between eicosanoids and TGF-β1 produced by alveolar macrophage, the modification of TXB2 and PGE2 production by TGF-β1 in *in vitro* silica-exposed rat alveolar macrophages was investigated. We observed decreased production of TXB2 in the presence of anti-TGF-β1 antibodies, or high concentration (50 ng) of TGF-β1. TXB2 production increased in the presence of a low concentration of TGF-β1 (2 ng) in the culture medium. PGE2 production increased in...
the presence of anti-TGF-\(\beta_3\) antibodies. Exogenous TGF-\(\beta_3\) at low or high concentration did not have any effect on PGE\(_2\) production in rat alveolar macrophages.

**Materials and Methods**

**Materials:** Hanks' solution and Dulbecco's phosphate buffered saline (DPBS), and HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) buffer were prepared in our laboratory. Six-well tissue culture plates were obtained from Falcon Laboratory Products (Beckton and Dickinson Labware, Lincoln Park, NJ). DMSO (dimethylsulfoxide) was obtained from Calbiochem (San Diego, CA). Allopurinol, oxypurinol, allopurinol riboside, Penn/Strep, M199 medium (without phenol red), and human serum albumin were all obtained from Sigma (St. Louis, MO). Human recombinant and polyclonal anti-TGF-\(\beta_3\) antibody were obtained from R&D Systems (Minneapolis, MN). RIA reagents for TXB\(_2\) and PGE\(_2\) assay were purchased from Advanced Magnetics, Inc. (Boston, MA).

**Isolation and culture of rat alveolar macrophages:** Sprague-Dawley rats weighing approximately 225 g were lavaged *ex vivo*. Rats were given an intramuscular injection of sodium pentobarbital (10 mg) and the rib cage was opened to expose the lungs. An incision halfway through the trachea was made and the lavage tube of the trachea cannula was inserted into the incision. The lungs were washed five times each with 10 ml of lavage fluid (phosphate-buffered saline containing 0.1% EDTA, at 37°C). The collected lavage fluid was filtered through sterile Nitex gauze and centrifuged at 250 x *g* for 10 min. The supernatant was discarded, and the cells suspended in M199, 10 mM HEPES 0.3% human serum albumin, 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin (culture medium). Cells from all rats were pooled and centrifuged again at 250 x *g* for 10 min. Cells were enumerated in a haemocytometer. Viability was determined by the exclusion of trypan blue and cells were plated at 1 x 10^6/ml of culture medium in 6-well culture plates. The cells were preincubated for 1 h at 37°C in a humidified atmosphere at 5% CO\(_2\) in air. The nonadherent cells were removed by aspiration. Fresh culture medium (37°C) was placed in the wells and unlabelled arachidonic acid was added at a final concentration of 3 \(\mu\)M. This incubation with arachidonic acid was conducted in order to replenish endogenous arachidonic acid utilized during the exaggerated eicosanoid production which occurs during cell isolation and attachment. The cells were incubated at 37°C for 24 h. After incubation the medium containing arachidonic acid was removed and fresh culture medium was placed in the wells. Macrophages were activated with a microcrystalline form of silica dust (Min-U Sil, 2–7 \(\mu\)m diameter, provided by Dr Peter Bolsitis, MIT) at a final concentration of 200 \(\mu\)g/ml, at 37°C for 24 h, in the absence or presence of anti-TGF-\(\beta_3\) antibodies, or TGF-\(\beta_3\). Nonspecific antibody as an isotype-matched control for anti-TGF-\(\beta_3\) antibody was tested. Media were collected into silanized tubes for the determination of eicosanoids.

**Radioimmunoassay for TXB\(_2\) and PGE\(_2\):** Following incubation, the culture medium was removed and acidified to pH 3.0 with 1 N HCl. Eicosanoids were extracted twice with 2 ml ethyl acetate/cyclohexane (1:1). The extracts were stored at −20°C until analysis of eicosanoids by specific radioimmunoassay, as described previously. The antibodies utilized in the assay of TXB\(_2\) or PGE\(_2\) showed less than 1% cross-reactivity with other eicosanoids.

**Cell viability test:** The lactate dehydrogenase (LDH) activity in rat culture conditions was measured using a Sigma kit for LDH. There was no detectable LDH enzyme activity in the culture medium from rat alveolar macrophages.

**Data analysis:** Results are presented as means ± S.E. of a number of independent experiments.

**Results**

**The effect of TGF-\(\beta_3\) on TXB\(_2\) production in rat alveolar macrophages:** Table 1 shows the effect of anti-TGF-\(\beta_3\) antibodies, or low or high concentrations of TGF-\(\beta_3\), on TXB\(_2\) production in silica-exposed rat alveolar macrophages. In the presence of 5 \(\mu\)g of anti-TGF-\(\beta_3\) antibodies in the culture medium, during the process of macrophage activation with silica, the production of TXB\(_2\) was inhibited. The TGF-\(\beta_3\), at 2 ng concentration in the culture medium, potentiated TXB\(_2\) production. At 50 ng of TGF-\(\beta_3\) in the culture medium, the production of TXB\(_2\) was inhibited.

**The effect of TGF-\(\beta_3\) on PGE\(_2\) production in rat alveolar macrophages:** Table 2 shows the effect of anti-TGF-\(\beta_3\) antibodies, or low or high concentrations of TGF-\(\beta_3\), on PGE\(_2\) production in silica-exposed rat alveolar macrophage. In the presence of 5 \(\mu\)g of anti-TGF-\(\beta_3\) antibodies in the culture medium, the production of PGE\(_2\) was
Table 1. The effect of TGF-β1 on TXB2 production by rat alveolar macrophages exposed to silica dust

| Treatment          | Experiment 1            | Experiment 2            | Experiment 3            |
|--------------------|-------------------------|-------------------------|-------------------------|
|                    | TXB2 (pg/ml) | % Control | TXB2 (pg/ml) | % Control | TXB2 (pg/ml) | % Control |
| Control            | 62 ± 2        |          | 179 ± 5     |          | 79 ± 3       |          |
| Silica             | 132 ± 2       | 213      | 369 ± 2     | 206      | 172 ± 9      | 218      |
| Silica + 6 μg anti-TGF-β1 | 60 ± 5 | 100      | 313 ± 2     | 175      | 103 ± 7      | 130      |
| Silica + 2 ng TGF-β1 | ND      | ND       | 491 ± 3     | 274      | 219 ± 2      | 277      |
| Silica + 50 ng TGF-β1 | 47 ± 5 | 76       | 69 ± 0      | 39       | 168 ± 3      | 200      |

Alveolar macrophages at 1 × 10^6 cells were activated with 200 μg of silica dust in the absence or presence of anti-TGF-β1 antibodies, or TGF-β1 added at the time of cell activation, and incubated at 37°C for 24 h. TXB2 was assayed by the RIA as described in Methods. n = 2 in each experimental group. ND = not done.

Table 2. The effect of TGF-β1 on PGE2 production by rat alveolar macrophages exposed to silica dust

| Treatment          | Experiment 1            | Experiment 2            | Experiment 3            |
|--------------------|-------------------------|-------------------------|-------------------------|
|                    | PGE2 (pg/ml) | % Control | PGE2 (pg/ml) | % Control | PGE2 (pg/ml) | % Control |
| Control            | 445 ± 6        |          | 207 ± 9     |          | 411 ± 10     |          |
| Silica             | 939 ± 3        | 211      | 241 ± 10    | 116      | 438 ± 10     | 107      |
| Silica + 6 μg anti-TGF-β1 | 1137 ± 75 | 256      | 330 ± 9     | 159      | 783 ± 20     | 191      |
| Silica + 2 ng TGF-β1 | ND      | ND       | 253 ± 3     | 122      | 457 ± 2      | 111      |
| Silica + 50 ng TGF-β1 | 840 ± 91 | 189      | 227 ± 7     | 110      | 504 ± 20     | 123      |

Alveolar macrophages at 1 × 10^6 cells were activated with 200 μg of silica dust in the absence or presence of anti-TGF-β1 antibodies, or TGF-β1 added at the time of cell activation, and incubated at 37°C for 24 h. PGE2 was assayed by the RIA as described in Methods. n = 2 in each experimental group. ND = not done.

Potentiated. The addition of 2 ng of TGF-β1 to the culture medium had no apparent effect on PGE2 production in either of the two experiments performed. At 50 ng of TGF-β1, the production of PGE2 varied.

Discussion

This study was designed to evaluate the regulation of TXB2 and PGE2 production by TGF-β1 in alveolar macrophages. The reason for these studies was to supplement the limited information in the literature on the relationship between the inflammatory mediators released by a specific inflammatory cell. TXB2, PGE2 and TGF-β1 are the principal inflammatory mediators released by alveolar macrophages during the lung's defense process against pathogens or foreign matter. We have demonstrated in earlier studies that these three mediators are released from rat alveolar macrophages upon exposure to silica dust. In those studies we observed a striking difference between the kinetics of release of TGF-β1 (maximum release at 15 min post-silica dust) and TXB2 (plateau release at 6 h post-silica dust), or PGE2 release (plateau release at 6 h post-silica dust). Because it is thought that TGF-β1 has a central regulatory role in vascular physiology and pathology, we formulated a hypothesis that perhaps TGF-β1 has an impact on the production of either TXB2 or PGE2, or both, by alveolar macrophage upon silica dust-exposure.

We observed a positive effect of TGF-β1 on TXB2 production in rat alveolar macrophages which had been exposed to silica dust. In the absence of TGF-β1 (plus anti-TGF-β1 antibodies), the quantity of TXB2 released from rat alveolar macrophages to the culture medium in response to silica exposure was significantly smaller when compared to the response with silica alone. Exogenous TGF-β1, at low concentration, potentiated the action of silica dust, and the amount of TXB2 produced by alveolar macrophages was significantly greater than the amount of TXB2 released by alveolar macrophages activated by silica alone. High concentration of TGF-β1, however, inhibited TXB2 production in rat alveolar macrophages; in response to silica, the amount of TXB2 released was smaller than the amount of TXB1 produced in rat alveolar macrophages activated with silica alone, or in the absence of TGF-β1 (in the presence of anti-TGF-β1 antibodies). This observation suggests that upon exposure to silica dust a homeostatic autocrine loop for TGF-β1, that regulates the production of TXB2, appears to function in rat alveolar macrophages. Our data also suggest the role for concentration of TGF-β1 in the regulation of TXB2 production; depending on the concentration, this growth factor appears to be either stimulatory or inhibitory to rat alveolar macrophage, and to TXB2 production.

At the same time, however, the effect of TGF-β1 on the generation of another eicosanoid,
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namely PGE₂, in silica-exposed rat alveolar macrophages appeared to be very different to that observed for TXB₂. In the absence of TGF-β₁ (plus anti-TGF-β₁ antibodies), the amount of PGE₂ produced in silica-exposed alveolar macrophages was greater than when compared to silica alone. When anti-TGF-β₁ antibodies were present in the culture medium during the exposure of macrophages to silica, we observed that (1) these macrophages liberated greater amount of PGE₂ compared to silica alone; and (2) there was a stimulation of PGE₂ production by alveolar macrophages which did not produce PGE₂ after exposure to silica. Furthermore, the presence of exogenous TGF-β₁, at low or high concentrations, had either no apparent effect, or a variable effect, respectively, on PGE₂ production in silica-exposed rat alveolar macrophages when compared to silica alone. Our data suggest, therefore, a possible distinct biological role for endogenous, but not exogenous TGF-β₁ to down-regulate PGE₂ production in rat alveolar macrophages after exposure to silica.

Data presented in this manuscript provide evidence of the regulatory role of TGF-β₁ in the liberation of inflammatory eicosanoids, TXB₂ and PGE₂, in rat alveolar macrophages, when the cells are exposed to silica dust. In support of our observation are the reports of Datta et al., where the authors demonstrate that TGF-β₁ inhibits lipooxygenase and epoxyoxygenase eicosanoid production by osteosarcoma cells, or eicosanoid metabolism in osteogenic osteosarcoma cells. The regulation of eicosanoid liberation by TGF-β₁, or the regulation of TNF-α production by TGF-β₁ suggests that the mechanism of action of TGF-β₁ on tissues may not necessarily be direct, but rather it requires actions of other mediators. The interrelationship between TGF-β₁ itself and all the inflammatory mediators directed by TGF-β₁ in inflammation appears to be very intriguing.

In summary, our data suggest that in silica-exposed alveolar macrophages, both endogenous and exogenous TGF-β₁ regulate TXB₂ production. Our data suggest that endogenous, but not exogenous TGF-β₁ has a regulatory role in PGE₂ production. Because both TXB₂ and PGE₂ were measured in the same samples, and because there is a consistent increase in silica-stimulated PGE₂ synthesis in the presence of anti-TGF-β₁ antibodies in the absence of an effect of authentic TGF-β₁, it appears that in silica-exposed alveolar macrophages the regulatory effects of TGF-β₁ are different between the different components of the arachidonic acid pathway.

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