Lung fibroblasts inhibit activation-induced death of T cells through PGE₂-dependent mechanisms

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Yarovinsky, Timur O., and Gary W. Hunninghake. Lung fibroblasts inhibit activation-induced death of T cells through PGE₂-dependent mechanisms. Am J Physiol Lung Cell Mol Physiol 281: L1248–L1256, 2001.—Activation-induced cell death (AICD) is a regulatory mechanism eliminating excess activated T cells, mainly through a Fas/Fas ligand-dependent mechanism. The goal of this study was to determine whether mouse primary lung fibroblasts are capable of modulating AICD. Using T cell hybridoma DO11.10, we found that fibroblasts in coculture rescue T cells from AICD. Fibroblast-conditioned medium (FCM) also inhibited apoptosis of T cells activated with immobilized anti-CD3 antibody. The effects of lung fibroblasts are mediated, in part, by secreted prostaglandin E₂ (PGE₂) because treatment of fibroblasts with indomethacin decreased antiapoptic activity of FCM. Addition of exogenous PGE₂ to FCM from fibroblast cultures treated with indomethacin restored the inhibitory activity of FCM. Expression of Fas receptor and Fas ligand by anti-CD3-activated DO11.10 cells was not affected by PGE₂. However, the same concentrations of PGE₂ significantly downregulated activation of caspase-8 and caspase-3. These results demonstrate that lung fibroblasts inhibit the AICD of T cells by secreting PGE₂, which downregulates caspase activation and apoptosis.

T lymphocytes; apoptosis; prostaglandins; Fas ligand; caspases

ADAPTIVE IMMUNE RESPONSES in the lung evoke a dramatic expansion of T cell populations. The immune system must eliminate many of these activated T cells to confine the magnitude of the immune response, maintain clonal representation diversity, and restore the initial cell numbers after elimination of the foreign antigen (21). Failure to control the number of activated T cells may result in an excess of inflammation and harmful outcomes in the lung and is linked to such disorders as sarcoidosis, pulmonary fibrosis, and asthma.

One of the key mechanisms in immune regulation is a process commonly called activation-induced cell death (AICD). During this form of programmed cell death, the excess activated T cells are removed, mainly through Fas/Fas ligand (FasL) and tumor necrosis factor-dependent killing (21). Resting T cells are resistant to apoptosis after the initial T cell receptor (TCR) stimulation but become very sensitive after activation and proliferation in the presence of interleukin (IL)-2. In this setting, reengagement of the TCR leads to expression of FasL and autocrine or paracrine death of T cells through Fas receptor signaling. It has been shown that lung lymphocytes are eliminated through this pathway in vivo during responses to inhaled particulate antigen (29). The other mechanism for regulating the number of activated T cells is lymphokine withdrawal or passive apoptosis, which removes activated proliferating T cells when they lack stimulation through the γ-chain of the IL-2 receptor (1).

There is accumulating evidence that the pulmonary microenvironment participates in tight regulation of immune responses in the lungs. Alveolar macrophages play a major role in regulating T cell functions in the pulmonary environment (41). Recent reports (12, 37) suggest that lung fibroblasts may also contribute to the regulation of immune responses by secreting chemokines, expressing adhesion molecules, or directly interacting with lymphocytes. Moreover, fibroblasts were shown to rescue T cells from lymphokine withdrawal apoptosis, mainly through type I interferons (32).

The goal of this study was to determine whether lung fibroblasts are capable of modulating AICD. We demonstrate that mouse primary lung fibroblasts inhibit AICD of T cell hybridoma DO11.10 through secretion of prostaglandin E₂ (PGE₂). PGE₂ does not mediate this effect on DO11.10 cells by downregulating FasL expression but rather inhibits caspase-8 activation and prevents apoptosis downstream of the Fas receptor.

MATERIALS AND METHODS

Reagents and Antibodies

BSA, concanavalin A (ConA), phorbol 12-myristate 13-acetate (PMA), and ionomycin were purchased from Sigma (St. Louis, MO). Polystyrene 6-μm micro beads were acquired from Polysciences (Warrington, PA). PGE₂ was obtained from Cayman Chemical (Ann Arbor, MI). Tissue culture plates and flasks were from Corning Costar (Corning, NY). All media and reagents for cell culture were obtained from Life Technologies (Rockville, MD) unless otherwise noted.

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Low-endotoxin and azide-free hamster anti-mouse CD3e (145–2C11), R-phycocerythrin-conjugated anti-Fas (Jo2), anti-FasL (MFL3), isotype control, and FITC-labeled annexin V antibodies were purchased from BD PharMingen (San Diego, CA).

Cells

Mouse lung fibroblasts. Whole lungs were removed from exsanguinated female C57BL/6 mice (8–12 wk old), transferred to Dublecco's PBS, minced into 2- to 3-mm pieces, and subsequently treated with dispase (1 U/ml) and 0.25% trypsin with 1 mM EDTA. Cell suspensions were filtered through 70-μm nylon cell strainers (Corning Costar), washed, and resuspended in RPMI 1640 medium supplemented with 10% FCS, nonessential amino acids, 2 mM l-glutamine, 5 × 10⁻⁵ M β-mercaptoethanol (Sigma), and 50 μg/ml of gentamicin (Mediatech, Herndon, VA), henceforth referred to as complete medium. Cells were grown at 37°C in a humidified 5% CO₂ atmosphere in T75 cell culture flasks and passaged every 5–7 days by dissociation of confluent cultures with 0.25% trypsin and 1 mM EDTA by the splitting of cell cultures 1:2. The primary cultures were characterized by staining with antibodies for fibronectin, CD45, and cytokeratin at passage 3. More than 90% of the cells were morphologically consistent with fibroblast phenotype and stained positive for intracellular and pericellular fibronectin. Less than 5% of cells stained for cytokeratin, and no cells stained for CD45. The fibroblasts were used in experiments between passages 3 and 8.

DO11.10 hybridoma T cells. DO11.10 hybridoma T cells were kindly provided by Dr. Barbara Osborne (University of Massachusetts, Amherst, MA) and Dr. Philippa Marrack (National Jewish Medical and Research Center, University of Colorado, Denver, CO). Once received, the cells were expanded, divided into aliquots, and cryopreserved. Aliquots of DO11.10 cells were thawed, maintained in complete medium at concentrations of 10⁶ to 5 × 10⁶ cells/ml, and used within 2 wk to avoid changes during culture.

Fibroblast-Conditioned Medium

Mouse lung fibroblasts were allowed to grow in complete medium in T75 flasks for 4 days. The culture medium was replaced, the cells were washed twice with serum-free medium, and fibroblasts were cultured for 4 more days; conditioned cell medium was collected, filtered through 0.22-μm filters, and stored in aliquots at −70°C. In addition, fibroblast-conditioned medium (FCM) was collected from exponentially growing cultures. In some experiments, indomethacin (10 μg/ml; Sigma) dissolved in absolute ethanol or carrier alone (ethanol) was added to the medium. The final concentration of ethanol in the culture medium was 0.1%.

Concentrations of PGE₂ in the FCM were measured with a competitive enzyme immunoassay according to the manufacturer’s recommendations (Amersham Pharmacia Biotech, Piscataway, NJ).

Activation of T Cells in Coculture With Fibroblasts

Anti-CD3 or isotype control monoclonal antibody (MAb) was adsorbed to 6-μm microbeads by incubating 2 μg/ml of the MAb with 10⁷ microbeads for 1 h with constant mixing. The beads were washed twice with PBS, incubated for an additional hour in 1% BSA in PBS, washed again with PBS, and stored in 1% BSA in PBS until used. Before the experiments, the fibroblasts were seeded at 60 × 10⁵ cells/well in 24-well plates and allowed to grow to confluence for 3–5 days.

The wells with fibroblasts were gently washed twice with warm complete medium, and T cells were added to the fibroblast cultures or empty wells at 10⁵ cells/well with microbeads at 1:1. ConA (20 μg/ml) or a combination of PMA and ionomycin (50 ng/ml and 0.5 μg/ml, respectively) was added to the cultures as an alternative T cell activator. The cells were cocultured for 24 h and harvested by pipetting. Cell death was estimated by trypan blue exclusion and manual counting under the microscope.

Assessment of AICD

Tissue culture plates were coated with anti-CD3 or isotype control MAb (2.5 μg/ml in PBS) for 1 h at 37°C and washed with RPMI 1640 culture medium before use. T cells (10⁵/well) were added to the wells with different dilutions of FCM or other agents such as PGE₂ or indomethacin in a total volume of 100 μl and cultured for 8 h.

A water-soluble tetrazolium salt (WST-1, Roche Molecular Biochemicals, Indianapolis, IN) was used to determine cell death. Tetrazolium salts are reduced by cellular enzymes such as mitochondrial dehydrogenases only in live cells and are often used to measure cell death, viability, or proliferation. In contrast to other tetrazolium salts such as MTT, XTT, and MTS, WST-1 is more stable and yields a water-soluble product. A ready-to-use solution of WST-1 was added to the cultures (10 μl/well), and the absorbance of the wells was measured at 450 nm after 30 min of incubation at 37°C in a 5% CO₂ humidified atmosphere.

The percentage of cell death is expressed as [1−(mean optical density at 450 nm (OD₄₅₀) in triplicate wells with anti-CD3-stimulated cells]/[mean OD₄₅₀ in triplicate wells with isotype control MAb or nonstimulated cells] × 100. In the case of treatment with FCM, PGE₂, or indomethacin, the agents were added to the wells with nonstimulated and anti-CD3-activated T cells.

The percentage of cells undergoing apoptosis was determined by detection of phosphatidylserine expression on the outer surface of the cells. Briefly, cells were harvested, washed in staining buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 5 mM KCl, and 1.8 mM CaCl₂), and resuspended at 5 × 10⁵ cells in 100 μl. The cells were incubated with 5 μl of FITC-labeled annexin V for 15–20 min in the dark, and the percentage of annexin V-positive cells was measured by flow cytometry after the addition of 400 μl of the staining buffer.

Apoptosis was also measured by DNA fragmentation assay. T cells (10⁵/sample) were harvested 8 h after stimulation, washed twice in PBS, and fixed in cold 70% ethanol overnight. After two washes in PBS, the cells were treated with 0.25 mg/ml of RNase A (Roche Molecular Biochemicals) and stained with 50 μg/ml of propidium iodide at 4°C for 30 min in the dark and analyzed for DNA content. The cells from the sub-G₀/G₁ peak were considered apoptotic.

Staining for Surface Expression of Fas and FasL

Anti-CD3-stimulated and control T cells (5 × 10⁵) were harvested, washed in PBS, and resuspended in 100 μl of staining buffer (3% FCS and 0.1% sodium azide in PBS). The cells were incubated with 1 μl of anti-Fas, anti-FasL, or isotype-matched antibodies labeled with R-phycocerythrin for 30 min, washed twice with the staining buffer, and analyzed by flow cytometry.
**Measurement of Caspase-8 and Caspase-3 Activity in Activated T Cells**

CaspTag caspase activity kits (Intergen, Purchase, NY) were employed to measure activation of caspases in T cells. The kits are based on inhibitors with a high affinity for caspase-8 [carboxyfluorescein-Leu-Glu-Thr-Asp-fluoromethylketone (FAM-LETD-FMK)] and caspase-3 [carboxyfluorescein-Asp-Glu-Val-Asp-fluoromethylketone (FAM-DEVD-FMK)]. The inhibitors are cell permeable because of the presence of fluoromethylketone and bind irreversibly to the active site of the caspase enzyme. Washing off the unbound inhibitors allows detection of cells containing active caspases because of the presence of carboxyfluorescein in the bound inhibitors.

The inhibitors were added to the wells at a 1:150 final dilution 5 h after stimulation with immobilized anti-CD3 MAb. The cells were cultured for an additional hour, harvested, washed twice with the provided buffer, and immediately analyzed by flow cytometry.

**Flow Cytometry Analysis**

A total of 10,000 cells that satisfied a gate on forward and side scatter to eliminate aggregates and debris was acquired with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Data analysis was performed with WinMDI 2.8 software (Joseph Trotter, Scripps Institute, La Jolla, CA). Samples stained with isotype-matched control antibodies were used as negative controls to determine the percentage of FasL-positive cells. Expression of Fas receptor was measured as the geometric mean channel fluorescence of nonstimulated and anti-CD3-activated cells. The samples with nonstimulated cells served as the negative control for measuring activities of caspase-8 and caspase-3.

**Statistical Analysis**

The results are expressed as means ± SE. The amount of PGE2 in FCM from growing versus confluent cultures was compared with an unpaired t-test. ANOVA for repeated measures was employed to analyze the rest of the data. If a significant difference was found (P < 0.05), the individual groups were compared with the control group with the use of Dunnett’s test or compared with each other with the Bonferroni test. All calculations were performed with GraphPad Prism software version 3.0 (GraphPad Software, San Diego, CA).

**RESULTS**

**Coculture With Fibroblasts Prevents AICD of T Cells**

We employed the model of T cell hybridoma DO11.10 to investigate potential effects of lung fibroblasts on AICD in vitro. This cell line expresses a TCR specific to chicken ovalbumin peptide OVA233–339 (10) and undergoes FasL-dependent apoptosis when stimulated through TCR or with immobilized anti-CD3e-chain antibody (46). DO11.10 is one of the most characterized T cell line models for studying AICD (23, 34, 42, 43). We used an anti-CD3 MAb immobilized on microbeads to stimulate T cells cultured alone or in direct coculture with mouse primary lung fibroblasts (Fig. 1A). It has been shown previously that major histocompatibility complex microbeads coated with class I proteins or the MAb against CD3e-chain provide adequate stimulation signal to T cells through TCR and serve as a convenient tool for studying T cell activation in coculture models (28). The microbeads coated with anti-CD3 MAb killed >50% of the T cells cultured alone after 24 h of incubation. In contrast, when T cells were cocultured with fibroblasts, AICD was completely prevented. Treatments with ConA or a combination of PMA and ionomycin, which mimic TCR stimulation through direct activation of protein kinase C (PKC) and an increase of calcium, are effective inducers of cell death in activated proliferating T cells (16). As expected, both treatments caused AICD in T cells cultured alone. Coculture with fibroblasts significantly decreased cell death when T cells were stimulated with ConA or PMA-ionomycin (Fig. 1B).

The decrease in the percentage of dead T cells cocultured with fibroblasts was not due to the uptake of apoptotic cells by fibroblasts because the total number of T cells recovered was not different between T cells cultured alone with fibroblasts. Thus mouse primary lung fibroblasts are capable of inhibiting cell death induced through TCR or by direct activation of PKC and an increase of calcium.

**Fibroblasts Secrete Factors That Inhibit AICD**

We explored whether the AICD-inhibiting effect of fibroblasts could be mediated by secreted factors. Cell-free supernatants were collected from fibroblast cultures and added to T cells stimulated with plate-bound anti-CD3 MAb (Fig. 2). We found that FCM inhibits AICD in a dose-dependent manner (Fig. 2A). Inhibition of AICD was apparent when FCM was used at a 1:8 dilution and was even more profound at 1:4 and 1:2 dilutions. We used the 1:2 dilution in the following experiments.
Cell death evoked by TCR activation, as well as apoptosis in general, is characterized by a distinctive increase of phosphatidylserine expression on the outer leaflet of the plasma membrane, which can be detected by the binding of fluorescently labeled annexin V (44).

We determined whether FCM decreased T cell death due to inhibition of apoptosis. Addition of FCM significantly decreased the percentage of annexin V-binding T cells, suggesting that FCM decreased anti-CD3-induced apoptosis (Fig. 2, B and C).

Similar results were obtained when DNA fragmentation, another marker of apoptosis, was monitored (Fig. 3). DNA fragmentation was apparent in more than one-third of T cells as early as 8 h after stimulation with anti-CD3 MAb. FCM added to the cultures inhibited DNA fragmentation almost fourfold. Negligible DNA fragmentation was observed when T cells were incubated with isotype control antibody. Taken together, these data demonstrate that fibroblasts secrete factors that inhibit AICD of T cells through prevention of apoptosis.

PGE2 Secreted by Fibroblasts Mediates Inhibition of AICD

Primary fibroblasts are known to secrete significant amounts of PGE2 into the culture medium (7, 49). PGE2 is known to be a potent modulator of T cell functions including proliferation, production of IL-2 and interferon-γ, differentiation, and AICD (2, 19, 33). To evaluate the role of PGE2 in the inhibition of AICD by FCM, we estimated secretion of PGE2 into the culture supernatant of confluent and exponentially growing lung fibroblasts. No significant difference in the levels of PGE2 in the conditioned medium from

![Figure 2](http://ajplung.physiology.org/)

**Fig. 2.** Fibroblast-conditioned medium (FCM) inhibits AICD. T cells were treated with immobilized anti-CD3 or isotype control antibody in the presence of FCM. A: inhibition of AICD by FCM was dose dependent as measured by cell death assay with reduction of WST-1, percent of apoptotic annexin V positive cells is shown. B and C: FCM at 1:2 dilution inhibited apoptosis of anti-CD3-activated T cells. No. of apoptotic T cells was measured by annexin V binding and flow cytometry in cultures with medium and FCM. Horizontal lines, percent of apoptotic annexin V-positive cells. Representative data (B) and means ± SE of 3 (A) and 6 (C) experiments are shown. Significantly different from T cell cultures without FCM; *P < 0.05; **P < 0.01.

![Figure 3](http://ajplung.physiology.org/)

**Fig. 3.** FCM inhibited DNA fragmentation in T cells stimulated by plate-bound anti-CD3 antibody. No. of apoptotic (sub-G₀/G₁) cells with fragmented DNA was determined by flow cytometry analysis of DNA staining with propidium iodide (PI). FL2-H, fluorescence intensity for DNA content, red channel. Horizontal lines, percent of cells with fragmented DNA. T cells were incubated with medium alone or FCM. Representative data (A) and means ± SE (B) of 3 experiments are shown. Significantly different from T cell cultures without FCM, ***P < 0.001.
growing versus confluent fibroblasts was found (growing, 31 ± 8 nM, n = 4 cultures; confluent, 26 ± 8 nM, n = 6 cultures). The addition of indomethacin (10 μg/ml) to the cultures decreased production of PGE2 to undetectable levels (sensitivity of the assay, <1 nM). Thus primary lung fibroblasts used in the study produced significant amounts of PGE2, and indomethacin, a nonspecific inhibitor of cyclooxygenases, could extinguish PGE2 from culture supernatants.

To evaluate the effect of fibroblast-produced PGE2 on prevention of AICD, we performed the following experiment. Confluent fibroblasts were cultured with and without indomethacin, cell culture supernatants were collected, and T cell death was assessed after stimulation with anti-CD3 MAb in the presence of FCM. PGE2 is known to modulate fibroblast proliferation and collagen production (6, 26, 31). To minimize potential interference of these effects, we used fibroblast cultures that reached confluence before indomethacin treatment and collection of supernatants. The addition of indomethacin to the fibroblast cultures significantly decreased the ability of FCM to prevent AICD (Fig. 4A). This suggests that inhibition of AICD by FCM is, in part, PGE2 dependent. However, the fact that indomethacin did not block all the protective effects of the FCM also suggests that other factors may play a role in this inhibition.

To exclude the possibility that carryover of indomethacin from fibroblast cultures could be the reason for the observed difference between conditioned medium from control and indomethacin-treated fibroblasts, we studied the effects of indomethacin on AICD. No significant changes in T cell death were observed when indomethacin was added to the medium (data not shown). Addition of physiological concentrations of PGE2 restored the inhibitory activity of conditioned medium from fibroblasts treated with indomethacin (Fig. 4B). Moderate but significant inhibition of AICD was also observed when exogenous PGE2 alone was added to the T cell cultures; cell death decreased from 58.2 ± 9.2% in control cultures to 45.7 ± 8.8% in the presence of PGE2 at a concentration of 100 nM (P < 0.01).

Furthermore, indomethacin-FCM protected T cells from DNA fragmentation less efficiently than FCM from control cultures (Fig. 4C). Addition of exogenous PGE2 (100 nM) to indomethacin-FCM completely blocked the DNA fragmentation induced by anti-CD3 MAb. These results suggest that inhibition of AICD by lung fibroblasts is at least partially mediated by PGE2 secreted from fibroblasts.

**Effects of FCM and PGE2 on Surface Expression of Fas and FasL by Activated T Cells**

It has previously been shown that AICD of T cell hybridomas depends on Fas-FasL interactions (3, 17). TCR stimulation induces Fas and FasL expression and renders activated cells susceptible to AICD (3, 17). First, we explored whether FCM or PGE2 modulates anti-CD3-induced expression of Fas receptor (Fig. 5).

Basal expression of Fas is practically undetectable; this is also correlated with the fact that unstimulated DO11.10 cells are resistant to treatment with anti-Fas antibodies (48). Most T cells expressed Fas after stimulation with plate-bound anti-CD3e antibody for 8 h. FCM did not have any effect on expression of Fas (Fig. 5). PGE2 at concentrations of 10 and 100 nM did not inhibit expression of Fas by T cells (data not shown).
Next, we determined the effects of FCM and PGE2 on the expression of FasL (Fig. 6). A fraction of T cells (35.7 ± 0.8%; n = 5 cultures) were positive for FasL staining after stimulation for 8 h. In our hands, longer incubation times led to massive cell death, complicating detection of fluorescently labeled cells. FCM significantly decreased the number of FasL-positive cells, whereas PGE2 at concentrations of 10 and 100 nM did not influence FasL expression.

Thus FCM had no effect on Fas receptor expression, but it contained a factor(s) distinct from PGE2 that inhibited AICD of T cells through downregulation of FasL expression. On the other hand, PGE2 alone at concentrations of 10–100 nM affected neither Fas receptor nor FasL expression in T cells, although it inhibited their AICD.

**PGE2 Inhibits Caspase-8 and Caspase-3 Activation in T Cells**

In the absence of significant effects of PGE2 on Fas or FasL expression, we analyzed signaling downstream of the Fas receptor to determine the potential mechanisms of the effects of PGE2 on AICD. Signaling through the Fas receptor leads to formation of death-inducing signaling complex (DISC) through recruitment of the adaptor proteins Fas-associated death domain and procaspase-8 (35). The interactions of the proteins in DISC result in autocleavage of procaspase-8 and the release of the active forms of caspase-8 (35). We used the fluorescent cell-permeable inhibitor of caspase-8, FAM-LETD-FMK, to investigate the effects of PGE2 on the activation of caspase-8 (Fig. 7). Less than 3% of unstimulated T cells were positive for caspase-8, whereas treatment of the cells with plate-bound anti-CD3 MAb led to activation of caspase-8 in >40% cells after 6 h. PGE2 inhibited caspase-8, and the effect was statistically significant at both concentrations used (10 and 100 nM). Therefore, PGE2 at physiological concentrations inhibited caspase-8 without downregulation of Fas and FasL expression.

In most T cells, active caspase-8 directly cleaves and activates caspase-3, which is considered the effector molecule that induces apoptosis (35). We used the fluorescent cell-permeable inhibitor of caspase-3, FAM-DEVD-FMK, to examine if the inhibitory effects of PGE2 on caspase-8 were further relayed to caspase-3 (Fig. 7). PGE2 significantly inhibited caspase-3 at concentrations of 10 and 100 nM. Thus caspase-8 may be inhibited by PGE2 in T cells, which prevents activation of caspase-3 and AICD.

**DISCUSSION**

The results of this study clearly demonstrate that mouse primary lung fibroblasts are capable of inhibiting AICD. The effect was mediated by secreted factors, among which PGE2 appears to be a major effector. The novel observation of this study is that PGE2 did not downregulate the expression of Fas or FasL. Rather, it suppressed AICD downstream of the Fas receptor by inhibiting activation of caspase-8. This observation provides one possible mechanism by which PGE2 inhibits AICD. These findings support the hypothesis that fibroblasts are not passive bystander cells fulfilling...
ing purely structural functions but that they actively participate in immune regulation (13).

A number of previous studies have shown interactions between lung fibroblasts and T cells. One study found that lung fibroblasts can be divided into two subsets that respond differently to interferon-γ and IL-4 (38, 39). These subsets of fibroblasts also differ in their expression of the Thy-1 marker (31). Lung fibroblasts derived from mice with T helper type 2 (Th2)-type granulomas produce more of the chemokine monocyte chemoattractant protein-1 than fibroblasts from mice with Th1-type granulomas (11). This release of monocyte chemoattractant protein-1 by lung fibroblasts might further polarize the immune response toward Th2 through increased production of IL-4 by CD4+ cells (12). Moreover, it was demonstrated that interaction of lung fibroblasts with T cells through CD40-CD40 ligand gives signals bidirectionally, being costimulatory to T cells and upregulating prostaglandin synthesis by fibroblasts (37, 49). Finally, fibroblasts were also shown to rescue T cells from cytokine deprivation apoptosis by secreting interferon-β (32).

The present study shows that lung fibroblasts can inhibit AICD, in part, by PGE2-dependent mechanisms.

Expression of the inducible form of cyclooxygenase-2 is a critical feature of inflammation. It plays a regulatory role in immune responses and controls fibroblast proliferation and synthetic activity through production of PGE2. It is thought that PGE2 serves as an anti-inflammatory and antifibrogenic agent in bleomycin-induced fibrosis (30). Decreased PGE2 secretion due to a defect in cyclooxygenase-2 induction has been associated with the pathogenesis of idiopathic and bleomycin-induced pulmonary fibrosis (45). The fact that PGE2 secreted by lung fibroblasts inhibits AICD suggests that PGE2 does not always inhibit immune reactions but may, in fact, sustain the presence of activated T cells in inflamed tissue.

The effects of PGE2 on T cells are almost exclusively attributed to signal transduction through the E-prostanoid receptors EP2 and EP4, which are positively coupled via G protein-mediated mechanisms to adenylate cyclase (9, 47). In previous reports (9, 33), it was shown that the inhibition of AICD of T cells by PGE2 is mediated by an increase of intracellular cAMP. The pharmacological activator of adenylate cyclase, forskolin, as well as cell-permeable analogs of cAMP, mimicked the effects of PGE2 in inhibition of AICD (14, 15, 20). Moreover, vasoactive intestinal peptide and the structurally related peptide pituitary adenylate cyclase-activating polypeptide (PACAP) had similar effects on inhibition of AICD of lymph node T cells and the T cell hybridoma 2B4.11 through the cAMP-elevating receptor common PACAP/vasoactive intestinal peptide type 2 (5). All these studies were in agreement with the model in which activation of cAMP-dependent PKA leads to inhibition of the transcription factors required for FasL expression, which is critical for AICD. Although some of these studies were inconsistent regarding inhibition of nuclear factor of activated T cells (NF-AT) by PKA (5, 20), most of them were in accord with the view that PKA downregulates FasL expression through inhibition of NF-κB. In our study, PGE2 at concentrations of 10–100 nM had no detectable effect on FasL surface expression in DO11.10 cells. Presumably, DO11.10 cells are different in terms of their response to PGE2 from T cell hybridomas 2B4 and 10I and other cell lines that were used in previous studies (9, 14, 15, 33).

In addition to PGE2, FCM seems to contain another factor(s) that inhibits AICD. Treatment of fibroblasts

Fig. 7. Activation of caspase-8 and caspase-3 is inhibited by PGE2. T cells were stimulated with immobilized anti-CD3 MAb in the presence of PGE2. Cell-permeable inhibitors of caspase-8 [carboxyfluorescein-Leu-Glu-Thr-Asp-fluoromethylketone (FAM-LETD-FMK)] and caspase-3 [carboxyfluorescein-Asp-Glu-Val-Asp-fluoromethylketone (FAM-DEVD-FMK)] were added at 5 h poststimulation, and activation of caspases was analyzed by the accumulation of the fluorescent inhibitors with flow cytometry. Horizontal lines, percent of cells with green fluorescence above background, indicating active caspase-8 or caspase-3. Representative data (A) and means ± SE (B) of 6 experiments are shown. Significantly different from T cell cultures treated with anti-CD3 antibody alone: *P < 0.05; **P < 0.01.
with indomethacin completely blocked PGE$_2$ production. Nevertheless, conditioned medium from these cultures retained some protective activity. Moreover, FCM inhibited FasL surface expression, whereas PGE$_2$ did not. Type I interferon-α and β have previously been found to be major antiapoptotic mediators in FCM (25, 32). They were shown to induce Bcl-X$_L$ expression and inhibit caspase-3 activation and the subsequent activation and translocation of PKC-δ (32, 36). Type I interferons may account for the PGE$_2$-independent antiapoptotic activity of FCM.

Overall, from previous studies (9, 33) and our study, it appears that PGE$_2$ may decrease FasL expression in some T cells but not in others. The fact that PGE$_2$ did not inhibit FasL expression in DO11.10 cells allowed us to make the novel observation that PGE$_2$ has antiapoptotic effects downstream of Fas receptor signaling. We found that PGE$_2$ downregulated the activation of caspase-8 and caspase-3 in DO11.10 cells. Our observation differs from a previous study showing that increases of intracellular cAMP do not protect Jurkat T cells from Fas-induced apoptosis (14). However, it is worth noting that Jurkat cells are classified as type II apoptotic cells where apoptosis through the Fas receptor involves a mitochondrial loop for activation of caspase-8 and caspase-3 rather than a direct activation of caspase-8 at the DISC in type I apoptotic cells (35). Although Jurkat T cells probably serve as one of the most frequently studied models of Fas-induced apoptosis and AICD, peripheral T cells and most T cell hybridomas seem to be type I cells (27, 35). These variations in caspase activation mechanisms may explain the different sensitivity to inhibition of apoptosis by PGE$_2$.

It has previously been described that the effects of PGE$_2$ in T cells are mediated primarily by increases of cAMP and activation of cAMP-dependent PKA (2, 9, 47). Cytoprotective effects of elevated levels of cAMP have been extensively studied. It was demonstrated that Akt may be activated by PKA independently of the phosphatidylinositol 3-kinase pathway (8). Akt is considered a major cell survival mediator due to its ability to phosphorylate and inhibit several physiological substrates important for apoptosis such as Bad (Bcl-2/Bcl-X$_L$ antagonist that causes cell death), caspase-9, and glycogen synthase kinase-3 (reviewed in Ref. 4). Direct phosphorylation and inactivation of Bad by cAMP-dependent PKA was also shown to prevent cell death (24). However, other pathways might be involved in rescuing T cells from Fas-induced death because this form of apoptosis is not dependent on Bad (27). Activation of PKA by cyclic nucleotides has been recently described to inhibit apoptosis through another Akt-independent pathway (22). Phosphorylation of a cell cycle protein, p21$^{cip1/waf1}$, by PKA was shown to form the complex between procaspase-8 and p21 to resist Fas-mediated cell death (40). The fact that PGE$_2$ downregulated caspase-8 in DO11.10 cells suggests that PKA may inhibit apoptosis upstream of caspase-3 either through prevention of procaspase-8 recruitment to the complex of Fas receptor and Fas-activated death domain or through inhibition of caspase-8 activity. Structural homologs of procaspase-8, named dominant negative caspase 8 (FLICE) inhibitory protein (c-FLIP), were demonstrated to protect T cells from Fas-induced apoptosis due to their ability to prevent activation of caspase-8 at DISC (18). So far, there is no evidence that cAMP-dependent PKA regulates DISC formation or caspase-8 activity. It will be the subject of future studies to determine what mechanism is employed in inhibition of Fas signaling in T cells.

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