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Human Follicular Dendritic Cells Express Prostacyclin Synthase: A Novel Mechanism to Control T Cell Numbers in the Germinal Center

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Stromal cells in the lymphoid organs provide a microenvironment where lymphocytes undergo various biological processes such as development, homing, clonal expansion, and differentiation. Follicular dendritic cells (FDCs) in the primary and secondary follicles of the peripheral lymphoid tissues interact with lymphocytes by contacting directly or producing diffusible molecules. To understand the biological role of human FDC at the molecular level, we developed a mAb, 3C8, that recognizes FDC but not bone marrow-derived cells. Through expression cloning and proteome analysis, we identified the protein that is recognized by 3C8 mAb, which revealed that FDC expresses prostacyclin synthase. The 3C8 protein purified from FDC-like cells indeed displayed the enzymatic activity of prostacyclin synthase and converted PGH2 into prostacyclin. In addition, prostacyclin significantly inhibited proliferation of T cells but delayed their spontaneous apoptosis. These findings may help explain why T cells constitute only a minor population compared with B cells in the germinal center.


Materials and Methods

Abs and reagents

The mAb 3C8 was developed as previously described (21), purified, and conjugated with biotin using a biotinylation kit (Pierce) according to the manufacturer’s instructions. Abs and reagents used in this work were anti-

β-actin, goat-anti-mouse IgG1, streptavidin-FITC (DAKO), mouse IgG1, PGI2 (Sigma-Aldrich), alkaline phosphatase (AP)-conjugated goat anti-mouse IgG1, biotin-prostacyclin, rabbit anti-human PGI2 (Cayman Chemical), un-conjugated anti-human CD3 (64.1, Dr. Y. S. Choi, Ochsner Medical Foundation, New Orleans, LA), and HRP-conjugated anti-rabbit Ig (Pierce).

Culture of HK cells and T cells

HK cells were prepared and maintained as described by Kim et al. (11). Synovial fibroblasts were a kind gift from Dr. C.-S. Cho (Division of Rheumatology, Center for Rheumatic Diseases in St. Mary’s Hospital, Seoul, Korea). HK cells and fibroblasts were grown in RPMI 1640 (Invitrogen...
Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen Life Technologies), and 80 μg/ml gentamicin (Sigma-Aldrich). T cells were isolated from tonsillar mononuclear cells by rosetting with SRBC; the resulting cells containing >98% CD3+ cells as analyzed by FACSscan (BD Biosciences). T cells were cultured under the various conditions as described in the figure legends in the presence or absence of PG2 and beraprost. For the proliferation assay, 96-well flat-bottom microwell plates were coated with 10 μg/ml 64.1 Ab, followed by incubation with T cells for the indicated periods of time. The degree of cellular proliferation was measured by using cell-counting kit-8 reagent (Dojindo Laboratories) according to the manufacturer’s instructions. The cellular proliferation was also measured by pulsing with 0.5 μCi [3H]thymidine (DuPont NEN) during the last 16 h culture period. The cultures were harvested onto glassfiber filters, and [3H]thymidine incorporation was measured by a liquid scintillation counter (Packard). In the survival assay, T cell survival was measured with the cell-counting kit-8 reagent or by directly counting viable cells after trypan blue staining.

Expression cloning of 3C8 Ag
A Uni-ZAP XR expression cDNA library of human rheumatoid arthritis-positive synoviocytes was purchased from Stratagene. The cDNA library was immunoscreened according to the manufacturer’s protocols. Briefly, the library at the concentration of 1 × 10^7 plaques in 1 μl of SM buffer (100 mM NaCl, 10 mM MgSO4 , 50 mM Tris-HCl, pH 7.5) was preincubated with 600 μl of XL1-Blue Escherichia coli (OD600 = 0.5) resuspended in 10 mM MgSO4 for 15 min at room temperature. Then 7 ml of top agar was added, and the mixture was plated immediately into a 150-mm agar plate. Plates were incubated at 37°C until small size plaques appeared. Nitrocellulose membranes (Schleicher & Schuell) impregnated in 10 mM isopropyl β-D-thiogalactoside (USB) were overlaid on the agar surface for at least 5 h at 37°C. The membranes were removed, washed in TTNT (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), and blocked with 3% skim milk in TTNT for 1 h at room temperature. The membranes were then incubated overnight at 4°C with 3C8 ascites diluted 1/1000 in blocking buffer. After thorough washing three times for 10 min in TTNT, AP-conjugated goat anti-mouse IgG1 (0.3 mg/ml diluted 1/5000 in blocking buffer) was applied for 2 h at room temperature. The membranes were washed five times and preincubated in AP buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM MgCl2, 64.1 Ab, followed by incubation with T cells for the indicated periods of time. The degree of cellular proliferation was measured by using cell-counting kit-8 reagent (Dojindo Laboratories) according to the manufacturer’s instructions. The cellular proliferation was also measured by pulsing with 0.5 μCi [3H]thymidine (DuPont NEN) during the last 16 h culture period. The cultures were harvested onto glassfiber filters, and [3H]thymidine incorporation was measured by a liquid scintillation counter (Packard). In the survival assay, T cell survival was measured with the cell-counting kit-8 reagent or by directly counting viable cells after trypan blue staining.

Western blotting and immunoprecipitation for MALDI-TOF mass spectrometry (MALDI-TOF-MS) analysis
Western blotting was conducted as previously described (24). 3C8 Ags were immunoprecipitated and visualized on 10% SDS-PAGE gel by Coomassie blue-staining. The 3C8 Ag band was excised from the gel with a sterile scalpel, subjected to in situ digestion with trypsin, and analyzed by MALDI-TOF-MS. The peptide mass fingerprints obtained were allowed to search against a mammalian subset in the NCBI nonredundant protein database. Sequence alignments were conducted using the ClustalW program.

Measurement of PGIS enzymatic activity
To measure PGIS enzymatic activity of 3C8 Ags, 3C8-immunoprecipitate and its supernatant were incubated with PGH2 for 30 min. The produced amount of 6-keto-PGF1α, chemically stable metabolite of prostacyclin, was measured using an enzyme immunoassay kit (Cayman) according to the manufacturer’s instructions.

Preparation of PGIS small interfering RNA (siRNA) duplexes and transfection of HK cells
In the survival assay, T cells were cultured with HK cells that were transfected with PGIS-specific siRNA. The construction of siRNA was conducted according to the manufacturer’s instructions (Ambion). For the construction of PGIS siRNA, sense-(5'-AAAGTTCTCTAGACACACACCTTGGTCTCT-3') and antisense-(5'-AAATTTGTGCGAGGAGACCTTAGACC-3') oligonucleotides were synthesized. The sequences of control siRNA with random PGIS nucleotides are as follows; sense- (5'-AACTGACTCTCTTCACCGACACACTTCTCT-3') and antisense-(5'-AAAGTTGTTGCTGTCTTACCGACACACTTCTCT-3'). These siRNAs were transfected into HK cells by using FuGENE6 transfection reagent (Boehringer Mannheim) according to the manufacturer’s directions. The degree of gene silencing was assayed by Western blotting.

Results
3C8 Ag is prostacyclin synthase
Because the 3C8 Ag was expressed in synovial fibroblasts as well as in FDC (24, 25), we attempted to isolate 3C8 cDNA from the synoviocyte cDNA library by expression cloning. A 3.2-kb cDNA insert was obtained from a positive plaque (Fig. 1). The sequence of the 3C8 cDNA insert matched exactly that of PGIS (data not shown).

To rule out the possibility that 3C8 Ab bound the isolated cDNA clone by cross-reactivity, we purified 3C8 protein from HK cells by immunoprecipitation for biochemical analysis of the 3C8 Ag.

DNA sequencing and identification of the clone
Sequencing of the insert cDNA was performed using a BioDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) and an automatic sequencer ABI 3730xl DNA Analyzer (Applied Biosystems). The sequencing primers used were the standard pBluescript/prl (5'-AATAGACCTCATATAAGG-3') and T7 (5'-AATACGACTCATACTAGTTG-3') universal primers and the internal custom-synthesized primer (5'-GATGTCTTCACACCTTTCCTGCG-3'). For identification, sequences were manually edited to remove vector sequences and compared with the National Center for Biotechnology Information (NCBI) nonredundant GenBank nucleotide database. Sequence alignments were conducted using the ClustalW alignment procedure (28) with the BioEdit software.

FIGURE 1. Expression cloning with cDNA library of synovial fibroblasts reveals the molecular identity of the 3C8 Ag. With a cDNA library of human rheumatoid arthritis positive synoviocytes, expression cloning was conducted by immunoscreening with 3C8 mAb. A positive plaque (A) was subjected to the second (B) or third (C) rounds of immunoblotting. A replica of B was immunoblotted with isotype-matched control Ab (D).
SDS-PAGE followed by immunoblotting with 3C8 Ab revealed two specific bands (Fig. 2A), a major and a minor band with molecular masses of around 55 kDa. The nominal molecular mass of PGIS is 57 kDa. These two specific bands were identified both as PGIS by MALDI-TOF-MS analysis (Fig. 2B and C), confirming the expression cloning result. The appearance of 3C8 as a doublet may have been resulted from protein fragmentation during immunoprecipitation procedure since a single band was obtained with Western blot analysis that was performed with 3C8 Ab and commercially available polyclonal Ab against PGIS (Fig. 3A). Immunoprecipitation with 3C8 Ab followed by immunoblotting with PGIS polyclonal Ab further confirmed that 3C8 mAb and anti-PGIS polyclonal Ab recognize the same molecule (Fig. 3B). Finally, because 3C8 Ag was successfully isolated in pure forms by immunoprecipitation, we investigated whether the isolated Ag would exhibit the enzymatic activity of PGIS, that is, conversion of PGH₂ into PGI₂. After incubation of 3C8 Ab with HK cell lysates followed by precipitation with protein A-Sepharose beads, we measured the PGIS activity of precipitates and supernatants. Compared with the control precipitate that had been incubated with isotype-matched control Ab, the 3C8 precipitate converted 15 times more PGH₂ into PGI₂ (Fig. 4A). At the same time, the PGIS activity of the control supernatant that had been incubated with control Ab was comparable to that of 3C8 precipitate while 3C8 supernatant lost most of the PGIS activity after incubation with 3C8 Ab (Fig. 4A). The production of PGI₂ by 3C8 precipitate was dependent on the concentration of PGH₂. However, increased addition of PGH₂ to control precipitate did not result in a significant increase of PGI₂ production (Fig. 4B). These results suggest that the protein that precipitated with 3C8 Ab is indeed the enzyme PGIS.

**Prostacyclin inhibits the proliferation of T cells but promotes their survival**

Widespread distribution of PGIS expression in the GC suggests its critical role in the GC reaction. The report that indomethacin reversed FDC-induced inhibition of T cell proliferation (12) prompted us to investigate the effect of prostacyclin on T cell proliferation. Because prostacyclin is a very unstable molecule (29),
we also examined the effect of beraprost, a more stable prostacyclin analog. T cells were stimulated to proliferate on the plate coated with anti-CD3 Ab and continuously proliferated up to 80 h poststimulation (Fig. 5A). Addition of prostacyclin resulted in a slight decrease of T cell proliferation, whereas beraprost significantly reduced the proliferation from 48 h (Fig. 5C). The inhibition of T cell proliferation was dose-dependent, which was more evident with beraprost than prostacyclin at the equivalent concentrations (Fig. 5, B and D). This inhibitory effect of prostacyclin and beraprost was confirmed because addition of beraprost gave rise to ~50% inhibition of [3H]thymidine uptake by proliferating T cells (Fig. 5E).

We next examined the effect of beraprost on T cell survival. T cells underwent spontaneous apoptosis, and only small numbers of viable cells were observed after 48 h of the culture. In contrast to its inhibitory effect on T cell proliferation, beraprost enhanced T cell survival during the 3-day culture period. The presence of beraprost resulted in significantly higher number of viable cells at 24 and 48 h (Fig. 6A). Beraprost enhanced the T cell survival in a dose-dependent manner, and significant difference was obtained at the concentration of 1 µM (Fig. 6B). Because HK cells express PGIS and beraprost enhances T cell survival, we investigated whether knockdown of PGIS from HK cells would modulate the effect of HK cells on T cell survival. The PGIS gene was silenced by transfection with siRNA against PGIS. The HK cells that were transfected with 100 nM PGIS siRNA expressed significantly reduced PGIS proteins, whereas endogenous expression of PGIS protein was slightly modulated by transfection with control siRNA (Fig. 6C). Freshly isolated T cells were cultured with equal numbers of normal or transfected HK cells. T cells cultured in the absence of HK cells exhibited 48% of viable cell recovery at the end of 48 h culture. Both normal HK cells and HK cells that were transfected with control siRNA increased the recovery to 57%. However, PGIS siRNA-transfected HK cells did not support the T

FIGURE 5. Prostacyclin and beraprost suppress T cell proliferation. Kinetics and dose response of the effect of PGI2 (A and B) or beraprost (C and D) on tonsillar T cell (1 × 10⁵ cells/well) proliferation stimulated by immobilized anti-CD3 Ab. The degree of cellular proliferation was measured colorimetrically by cell counting kit-8 at the indicated time points or at the end of 72 h culture (B and D). E. The effect of prostacyclin and beraprost on the [3H]thymidine uptake by proliferating T cells. Tonsillar T cells (1 × 10⁵ cells/well) were cultured in the presence or absence of prostacyclin or beraprost for 72 h, and [3H]thymidine incorporation during the last 16 h was measured. Results are presented as the mean ± SEM of three independent experiments. Asterisks indicate significantly different effect compared with controls (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
cell survival and yielded 49% of viable cell recovery (Fig. 6D). This result suggests that HK cells enhance T cell survival by producing prostacyclin. The effect of transfected HK cells on the T cell proliferation was not examined because the coating of culture plate with anti-CD3 Ab was technically problematic due to the presence of adherent HK cells.

Discussion

Based on the following results presented previously (25, 26) and in this paper, we conclude that human FDCs express prostacyclin synthase: 1) FDCs express 3C8 Ag. 2) Molecular cloning and proteome analysis reveal that 3C8 Ag is prostacyclin synthase. 3) 3C8 Ag is recognized by anti-PGIS polyclonal Ab. 4) Purified 3C8 protein displays PGIS activity.

Although PGs, particularly PGE2, have been suggested as potent modulators of immunity (30), their cellular source and biological functions at the culminating site of the immune response, the GC, were not studied. Strong expression of PGIS throughout the GC suggests that prostacyclin is produced by FDC and is the major PG species in this microenvironment. This is, to our best knowledge, the first report that FDC expresses PGIS. Therefore, 3C8 is the first mAb raised against human PGIS. Furthermore, the result that prostacyclin and beraprost inhibited T cell proliferation is extending the previous report by Butch et al. (12) that PGs are involved in FDC-mediated inhibition of T cell proliferation and specifies the molecular identity of the responsible PG. Our results may explain how T cell numbers are controlled in the GC. GC T cells are mostly CD4+ and specific to the inducing Ag (31, 32), suggesting cognate interactions between T and B cells. Considering that B cells are efficient APCs to T cells, it has been puzzling that GC T cells do not normally proliferate and are defective for IL-2 production in response to Ag stimulation (12, 33). Our current data and recent results by Tenner-Racz and colleagues (31) that showed 70–90% GC T cells express CTLA-4 suggest that the number of T cells in the GC appears to be tightly regulated by FDC and B cells. B cells may induce negative signals in T cells during the cognate interactions through CTLA-4. At the same time, FDCs in close contact with T cells inhibit T cell proliferation by producing prostacyclin.

FDC expression of PGIS is more evidence supporting the view that FDC originates from local fibroblast as reviewed by Heinen and Bosseloir (34). Ultrastructural (35, 36), enzymological (35, 37), and functional studies (38, 39) support this view. We demonstrated recently that fibroblasts as well as FDCs express 3C8 Ag (25). Because 3C8 Ag has turned out to be PGIS in the current study, fibroblast and FDC exhibit another common feature of expressing PGIS. Fibroblasts are the major producer of prostacyclin (40). When the requirement for FDC increases in the GC during immune response, fibroblasts in connective tissues may migrate to lymphoid follicles in response to certain chemokines and differentiate to FDCs as a result of interaction with GC B cells. FDC may produce prostacyclin, which controls the number of T cells by inhibiting their proliferation but promoting their survival. Lindhout et al. (41) have demonstrated that synovial fibroblasts could be maintained by human FDCs expressing a specific Ag, 3C8.
induced with IL-1β and TNF-α to express the FDC phenotype. Because fibroblasts are ubiquitous cells, it remains to be elucidated whether all kinds of fibroblasts are capable of differentiating to FDCs or whether this property is limited to certain types of fibroblasts. The distinct staining pattern of 3C8 Ab from those of other fibroblast-specific Abs (25) implies that only a subset of fibroblasts may differentiate to FDCs.

**Disclosures**

The authors have no financial conflict of interest.

**References**


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