Intestinal Fibroblast-Derived IL-10 Increases Survival of Mucosal T Cells by Inhibiting Growth Factor Deprivation- and Fas-Mediated Apoptosis[1]

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Mucosal T cells are essential to immune tolerance in the intestine, an organ constantly exposed to large amounts of dietary and bacterial Ags. We investigated whether local fibroblasts affect mucosal T cell survival, which is critical for maintenance of immune tolerance. Coculture with autologous fibroblasts significantly increased viability of mucosal T cells by inhibiting IL-2 deprivation- and Fas-mediated apoptosis, an effect that was both contact- and secreted product-dependent. Investigation of antiapoptotic factors in the fibroblast-conditioned medium (FCM) revealed the presence of IL-10 and PGE_2, but not IFN-β, IL-2, or IL-15. Although recombinant IFN-β, but not PGE_2, effectively prevented T cell apoptosis, neutralizing Ab studies showed that only IL-10 blockade significantly increased T cells apoptosis, whereas neutralizing IFN-β or IFN-α failed to inhibit the antiapoptotic effect of FCM. To confirm that fibroblast-derived IL-10 was responsible for preserving mucosal T cell viability, IL-10 mRNA was demonstrated in fibroblasts by Southern blotting and RT-PCR. When FCM was submitted to HPLC fractionation, only the peak matching rIL-10 contained the antiapoptotic activity, and this was eliminated by treatment with an IL-10-neutralizing Ab. Finally, when fibroblasts were transiently transfected with IL-10 antisense oligonucleotides, the conditioned medium lost its T cell anti-apoptotic effect, whereas medium from fibroblasts transfected with IFN-β antisense oligonucleotides displayed the same anti-apoptotic activity of medium from untransfected fibroblasts. These results indicate that local fibroblast-derived IL-10 is critically involved in the survival of mucosal T cells, underscoring the crucial importance of studying organ-specific cells and products to define the mechanisms of immune homeostasis in specialized tissue microenvironments like the intestinal mucosa. ***The Journal of Immunology,*** **2005, 175: 2000–2009.**

The gastrointestinal tract is essential to immune homeostasis, a function mediated by the lymphoid cells in the mucosal compartment (1). These cells must respond to the constant antigenic pressure of dietary and bacterial Ags. At the same time, they must maintain tolerance to avoid that the status of "physiological intestinal inflammation" is transformed into one of pathological inflammation (2). To perform these functions various systems must be in place, which may transcend the regulatory capacity of immune cells alone.

There is considerable evidence that, in most organs, local immune and nonimmune cells communicate closely with one another, exchanging reciprocal modulatory signals in both health and disease, and this is also true for the intestine (3). Among nonimmune cells, mesenchymal cells have received limited attention, although they exhibit a remarkable degree of phenotypic and functional plasticity (4). Pluripotent mesenchymal stem cells are found in the connective tissue of many developing organs (5), and in adult tissues they differentiate into heterogeneous populations of fibroblasts, which exert distinct functions in each organ system (6). This multiplicity of phenotypes and functions is also present in the intestine, where fibroblasts interact with surrounding cells, display distinctive secretory activities, and act as regulators of mucosal immunophysiology (7, 8).

Despite evidence indicating that intestinal fibroblasts interact with local T cells, the mechanisms of such interaction and their impact on regulation of mucosal immunity are unclear. Specifically, no study has investigated whether fibroblast-immune cell interactions in the intestine affect immunological memory or alter T cell survival, two functions that are critical to maintain tolerance and local immune homeostasis. The old dogma that T cell memory is lost unless there is continuous Ag exposure does not fit well in the context of the intestine, which is populated by a large pool of $CD45RO^+$ Ag-primed T cells, whose chance of re-encountering the original sensitizing Ag is unpredictable and sporadic (9). Additional environmental signals are necessary to preserve long-lived memory T cells in a quiescent state independently of TCR activation, but the nature of these signals is still poorly defined (10). Some studies have provided critical insights into novel pathways responsible for memory T cell survival and retention of Ag specificity, showing that stromal cells in the microenvironment exert a potent inhibitory activity on the death of neighboring T cells (11). Based on this evidence, we investigated whether intestinal fibroblasts can prolong the survival of mucosal T cells by inhibiting apoptosis, providing a possible mechanism for preservation of memory and tolerance in the intestine. The results of this study show that intestinal fibroblast-derived soluble factors promote survival of mucosal T cells by reducing T cell apoptosis triggered by

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growth factor deprivation or Fas ligation, and that IL-10 is the dominant factor responsible for the antiapoptotic effect of intestinal fibroblast-conditioned medium (FCM).

Materials and Methods

Isolation and culture of human intestinal fibroblasts (HIF)[3]

HIF were obtained as mucosal explants from surgically resected specimens of large and small bowel tissue from benign (colonic polyps, colonic inertia, and diverticular disease), neoplastic (colon cancer), or inflammatory diseases (Crohn's disease and ulcerative colitis) as described previously (12–14). HIF monolayers exhibited a typical fibroblastic cell morphology, which was readily distinguishable from that of epithelial cells, subepithelial myofibroblasts (15), or intestinal microvascular endothelial cells (16), and persisted throughout the life of the cultures. Routine immunofluorescence microscopy with a human α-smooth muscle actin mAb (Boehringer Mannheim) showed positive staining of cytoplasmic filaments; staining intensity was variable, but less intense than that seen in human aortic smooth muscle cells, but stronger than that of foreskin fibroblasts (both kindly provided by Dr. P. DiCorleto, Cleveland Clinic Foundation, Cleveland, OH). Staining for desmin was weakly positive, whereas staining for factor VIII-associated Ag (von Willebrand factor), PECAM-1 (PECAM-1/CD31), and an irrelevant control Ag (IgA2) was negative. Monolayers were totally free of leukocyte contamination (12).

Generation of HIF-derived conditioned medium

In all experiments involving cocultures with T cells, HIF were used between passages 3 and 5. For experiments using FCM for modulation of T cell survival, supernatants were collected from unstimulated HIF cultures between passages 1 and 5. In some experiments, FCM was derived from supernatants of HIF cultures stimulated with IL-1β (10 ng/ml; R&D Systems) or IFN-γ (500 U/ml; R&D Systems). When FCM was used for PG measurement, serum was omitted from the cultures. The concentration of IL-2, IL-10, IL-15, IFN-β, and PGE_2 in the FCM was measured using commercially available ELISA kits (R&D Systems for IL-2, IL-15, and PGE_2; BioSource International for IL-10; and TFB for IFN-β). In some experiments, the presence of IL-10 on the surface of HIF was investigated. Confluent unstimulated and IL-1β-stimulated HIF monolayers were detached by trypsin-EDTA (BioWhittaker-Cambrex BioScience), and individual cells were suspended at 1×10^6/ml in PBS with 2% FCS, stained with anti-IL-10 allophycocyanin (BD Pharmingen), and submitted to flow cytometric analysis using an isotype Ab for background control.

Establishment of mucosal T cell lines

Intestinal mucosal T cell lines were established from whole mucosal biopsies or from lamina propria mononuclear cells (LPMC) as described previously (17). Fresh biopsy specimens were incubated in the antibiotic mixture for 3–5 h, after which the tissue was rinsed, cut in 1- to 2-mm fragments, and placed in wells of tissue culture cluster plates with RPMI 1640 supplemented with 20% heat-inactivated FBS or 10% human AB serum, a mixture of penicillin, streptomycin, gentamicin, and Fungizone, 2.5% HEPES buffer, and 20 U/ml human rIL-2 (Cetus). T cell growth was noticed as early as 24 h, and IL-2-containing culture medium was exchanged every 3–4 days. Cell expansion was continued for ~2 wk when cells were harvested for coculture experiments. Some T cell lines were stored frozen in liquid nitrogen to allow growth of autologous HIF until these were obtained in sufficient numbers for coculture experiments.

LPMC were isolated as described previously (18). T cell lines were derived from LPMC stimulated by 0.02% PHA (Difco Laboratories) and 1 ng/ml PMA (Sigma-Aldrich). After 3 days, cells were washed, cultured, and maintained in the same medium used for biopsy-derived T cells, including 20 U/ml IL-2. Cultures were split every 3–4 days and recultured at 1×10^6 cells/ml.

After 2 wk of growth, analysis by flow cytometry revealed that cell lines contained $95.6 \pm 2.2\%$ $CD3^+$, $79.7 \pm 5.5\%$ $CD4^+$, $10.3 \pm 4.6\%$ $CD8^+$, $72.0 \pm 3.5\%$ HLA-DR^+, $35.3 \pm 4.3\%$ $CD25^+$, $95.2 \pm 3.6\%$ $TCR\alpha\beta^+$, and $1.4 \pm 0.5\%$ $TCR\gamma\delta^+$ cells, and were devoid of $CD19^+$ B cells and $CD14^+$ monocytes (17). The phenotype of the cell line was similar between biopsy- and LPMC-derived T cells. In some experiments, the IL-2-dependent mouse CTLL-N cell line was also used (19).

Fibroblast T cell cocultures

Confluent HIF were detached by treatment with a trypsin-EDTA mixture, washed, and seeded at 5×10^4 cells/ml HIF medium in wells of 24 cluster plates (Costar). After a 48-h incubation at 37°C, HIF reached confluency, and 1×10^6 T cells were added in a 2-ml total volume made up by equal proportions of HIF and T cell line medium. The optimal number of cells in each coculture was determined by preliminary experiments, where various numbers of HIF and T cells were mixed in different ratios (data not shown). Maximal protection against apoptosis was used as the criterion to define the optimal number of cells in the coculture. To assess the effect of HIF on viability and apoptosis of mucosal T cells, autologous cocultures were used. In this system, mucosal T cell lines were placed directly on confluent monolayers of autologous HIF, or physically separated from them by a porous membrane in a Transwell system (Costar), in the presence or absence of IL-2. After 7 days, the wells containing T cells mixed with HIF were swirled gently, and cells were harvested by three washings with PBS. In the Transwell system, T cells were harvested directly from the membrane-containing insert. All T cells were washed, and their degree of viability and apoptosis were measured as described below. This autologous T cell/HIF system was used only for these initial cell-cell contact and Transwell experiments.

Induction and modulation of T cell apoptosis

Mucosal T cell apoptosis was induced using various experimental conditions. In one set of experiments, T cells were cultured with 0.005% PHA, 100 ng/ml staphylococcus enterotoxin A (SEA; Toxin Technology), and 10 μg/ml immobilized anti-CD3 or anti-CD4 mAb (Ortho Diagnostics) for 18 h in the presence of IL-2. In another set of experiments, growth factor deprivation was used to induce apoptosis (17, 20). T cells grown in the presence of IL-2 for ~2 wk were washed extensively, and then incubated in medium alone for up to 48 h. Alternatively, mucosal T cell apoptosis was induced by ligation of the Fas pathway as reported previously (17). T cells were plated in wells of a 48-well cluster plate and incubated with anti-human Fas Ab (CH11; Medical & Biological Laboratories) at 100 ng/ml at 37°C.

To modulate T cell apoptosis, variable doses of rIL-2, IL-10 (R&D Systems), IFN-β, IFN-α (both from PeproTech EC), or PGE_2 (Cayman Chemical) were used. To determine the effect of soluble factors potentially produced by HIF, T cells were cultured with variable concentrations of FCM, which had or had not been pretreated by heating at 80°C for 1 h. FCM was generated from cultures of benign, neoplastic, or inflammatory disease tissue HIF, as described above, and T cells were not autologous to these HIF. In some experiments, neutralizing Abs (all at 10 μg/ml) were used against IL-10 (R&D Systems), IFN-β and IFN-α (Serotec). IL-10 and IFN-β isotype Abs were used as control. In some experiments, the effect of IL-2, IL-10, IFN-β, FCM on T cell proliferation was also measured by tritiated thymidine uptake.

Assessment of T cell viability and apoptosis

Cell death was monitored using a dye mix containing 100 μg/ml acridine orange and 100 μg/ml ethidium bromide (both from Sigma-Aldrich) in PBS. Apoptotic T cells (apoptotic bodies) were readily identified by a small size, round shape, very dense nucleus stained bright yellow by acridine orange, whereas in necrotic cells nuclear structure was preserved (21). The degree of apoptosis was quantified by an apoptotic index, calculated as percentage apoptotic cells divided by the number of total cells. In each culture, at least 200 cells were counted by two observers blind to the source of the cells. Interobserver variation averaged 2%. Viability was assessed by exclusion of 1% trypan blue exclusion method. Alternatively, apoptosis was additionally evaluated by FACS analysis of propidium iodide- or FITC-conjugated annexin V (BD Pharmingen)-stained cells.

Apoptosis was also assessed by DNA fragmentation examined by agarose gel electrophoresis. DNA was prepared from T cells as described with minor modification (22). In brief, T cells were washed in PBS and sedimented by centrifugation. Pellets were suspended in 500 μl of lysing buffer (10 mM EDTA, $1 \times$ SSC, containing sodium lauryl sarkosinate and proteinase K; both from Sigma-Aldrich) and incubated at 50°C for 2 h. The material was then ethanol-precipitated, mixed with loading buffer (10 mM EDTA, 40% sucrose, and 0.25% bromophenol blue), and then loaded into wells of a 1.5% (w/v) agarose gel containing 5 μg/ml ethidium bromide. Electrophoresis was conducted in 40 mM Tris-acetate, 1 mM EDTA (pH 8.0) at 90 V for 1 h. A typical fragmentation pattern showing 200 bp multiples indicated that apoptosis had occurred.

To investigate whether FCM differentially affected the survival of specific mucosal T cell subsets, flow cytometric analysis was used to measure

[3] Abbreviations used in this paper: HIF, human intestinal fibroblast; FCM, fibroblast-conditioned medium; LPMC, lamina propria mononuclear cell; SEA, staphylococcus enterotoxin A.

the relative proportions of $CD4^+$, $CD8^+$, and $CD25^+$ cells in T cells cultured in medium or 100% FCM, either deprived of IL-2 or treated with the CH-11 Ab.

Polymerase chain reaction

Total cellular RNA was extracted from HIF with RNAzol B (Cinna/Biotecx Laboratories), and cDNA was prepared using 50 ng of oligo(dT) primers, 1 μg of RNA, and 5 U of avian myeloblastosis virus reverse transcriptase (Stratagene) per reaction. IL-10 DNA was amplified from total cDNA in PCR (25 μl) using *Taq* polymerase buffer containing 20 mM each dNTP, 100 pmol of the indicated primers, template, and *Taq* polymerase (Stratagene). Reactions were run on a thermocycler (RoboCycler Gradient 40; Stratagene) for 30 cycles, the temperature profile consisting of 30-s denaturation at 94°C, 30-s annealing at 61°C, and 60-s extension at 72°C. The forward and reverse primers for IL-10 DNA amplification were 5′-AGCTCAGCACTGCTCTGTTGCC-3′ and 5′-CCTGAGGGTCTTCAGGTTCTC-3′, respectively (kindly provided by Dr. G. Mullin, North Shore University Hospital, Manhasset, NY), these oligonucleotides defining a 350-bp cDNA fragment. For IFN-β, the forward and reverse primers were 5′-ACTGCCTCAAGGACAGGATG-3′ and 5′-TAACCAGTCAGGCGGTTGAT-3′, which define a 400-bp product. Reactions were run for 35 cycles, with a temperature profile identical with that of IL-10. For β-actin, the oligonucleotide primers were 5′-GTGGGGCGCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′, which define a 550-bp product. Reactions were run for 26 cycles consisting of 30 s at 94°C, 60 s at 55°C, and 60 s at 72°C. Products resulting from IL-10 and β-actin amplification were run on 1% agarose gels, transferred to nylon membrane, and probed with a ^{32}P-labeled human IL-10 and β-actin cDNA probe. For negative control, RNA was omitted from cDNA synthesis and specific PCR amplification, whereas positive control consisted of RNA from freshly isolated LPMC.

In situ hybridization for IL-10 mRNA

Sense and antisense RNA probes for IL-10 were constructed with a combination of PCR technique and in vitro transcription as described previously (23). Briefly, SP6 RNA polymerase promoter sequence (GGATT TAGGTGACACTATA) was attached to the 5′ end of the forward primer (5′-GGATCCAAGCAAATGGCCAATGAG-3′) and reverse primer (5′-TGTTCTAAGCCAGAAACACTG-3′) for IL-10. An additional five nucleotides (GAATT) were introduced to the 5′ end of the promoter sequence to facilitate the binding of RNA polymerase. The PCR-amplified products from the cDNA of U937 cells (human histiocytic cell line) were electrophoresed and purified from the gel using Gene Clean Kit II (Bio 101) to use as the templates for in vitro transcription. Sense and antisense RNA probes were then synthesized with SP6 RNA polymerase, and their 3′ end was labeled with digoxigenin using the in vitro transcription kit (DIG RNA Labeling Kit; Boehringer Mannheim). After digestion of DNA templates with DNase, RNA probes were purified with phenol extraction and ethanol precipitation. In situ hybridization for detection of IL-10 mRNA was performed using the HIF monolayers. In brief, the formalin-fixed tissue sections were treated with 0.2 M HCl, permeabilized with proteinase K (50 μg/ml), and fixed in 4% paraformaldehyde. After dehydration with ethanol, the tissue sections were hybridized with digoxigenin-labeled IL-10 RNA probe. Thereafter, the hybridized probe was detected by incubating the sections with the dye substrates for antidigoxigenin alkaline phosphatase, 4-NBT, and 4-bromo-5-chloro-3-indolylphosphate. As negative controls, the reaction was processed without the probes, which consistently yielded negative results.

Microcapillary HPLC

FCM from HIF monolayers was subjected to differential precipitation with three volumes of absolute cold acetone on ice for 30 min, and the precipitate was dissolved in 10 ml of distilled water. After adding 1 ml of 100% trichloroacetic acid, the solution was kept on ice for 10 min, and the resulting precipitate was collected by centrifugation at 12,500 × *g* for 15 min. The precipitate was then rinsed with cold absolute acetone, and finally dissolved in 20 mM Tris-HCl (pH 8.0). Insoluble materials were removed by centrifugation at 12,500 × *g* for 15 min, and the supernatant was diluted with double distilled water and subjected to filtration through 0.22 = μm pore size filters (Millipore). The solution was immediately analyzed using reverse-phase capillary HPLC (Magic 2002; Michrom BioResources) (24). In brief, the protein fraction from culture supernatants was injected onto a reverse-phase capillary column (MagicMS C18, 1.0 × 150 mm, particle size 5 μm; Michrom BioResources), and was eluted with a 50-min gradient from 5 to 60% acetonitrile in 0.1% trifluoroacetic acid. The pump was operated at 50 μl/min.

HIF transfection studies

Subconfluent HIF monolayers were transfected with antisense oligodeoxynucleotides for IL-10 or IFN-β. The antisense sequence for IL-10 was 5′-GGTCTTCAGGTTCTCCCC-3′ (25), and that for IFN-β was obtained commercially (Biognostik). Cells were transfected using lipofectamine (Lipofectin; Invitrogen Life Technologies) according to the manufacturer's instructions. In brief, HIF were washed three times in DMEM and incubated at 37°C in a solution of Opti-MEM medium (Invitrogen Life Technologies) containing 5 μg/ml Lipofectin premixed with the 4 μg of antisense oligonucleotides. After a 3-h incubation, HIF were washed three times and further incubated in DMEM containing 5% FCS for 24 h. To confirm that the HIF monolayers expressed the transfected antisense oligonucleotide, in some experiments oligonucleotides were fluorescein-labeled before transfection, and the transfected cells were analyzed by fluorescence microscopy. In other experiments, the sequence used to detect IL-10 mRNA (see above) was amplified by RT-PCR.

Analysis of data

T cell lines and HIF were derived from histologically normal and inflamed mucosa. After examining 10 different T cell lines and 10 different HIF from normal and inflamed mucosa each, no disease-related differences were observed under all experimental conditions, and therefore data were analyzed together. Statistical analysis was performed using the paired *t* or Mann-Whitney *U* tests for parametric and nonparametric data, respectively. All results were expressed as mean ± SEM. Statistical significance was inferred at $p < 0.05$.

Results

Induction of mucosal T cell apoptosis

We initially investigated some of the pathways potentially involved in mucosal T cell apoptosis. IL-2 expanded, LPMC-derived T cell lines were exposed to various agents known to induce activation and apoptosis of peripheral blood T cells (26). Stimulation with PHA, SEA, and anti-CD3, but not anti-CD4, resulted in variable degrees of apoptosis, as shown by a characteristic DNA fragmentation pattern (Fig. 1*A*). This was accompanied by a proportional decrease in cell viability (Fig. 1*B*) and apoptosis as measured by propidium iodide or annexin V staining (data not shown). Next, we investigated the modulation of mucosal T cell apoptosis by IL-2. T cell lines were washed extensively and recultured with and without various doses of this cytokine. In the absence of IL-2, a marked loss of viability was observed (Fig. 1*D*), but even a small amount of IL-2 (2 U/ml) was sufficient to maintain excellent cell viability, which further increased with 20 U/ml. However, an additional increase in IL-2 concentration had some detrimental effect, because viability slightly diminished with 200 U/ml. The percentage viability was inversely but closely related to the degree of DNA fragmentation demonstrated by gel analysis (Fig. 1*C*) and the degree of apoptosis measured by propidium iodide- or annexin V-positive cells (data not shown). Finally, as previously reported (17), mucosal T cell apoptosis could also be induced by engagement of the Fas pathway (data not shown).

Modulation of mucosal T cell viability by HIF

After demonstrating that several stimuli could induce mucosal T cell apoptosis, we next investigated whether this phenomenon could be modulated by HIF. To do so, normal mucosa-derived, IL-2-driven T cell lines were cocultured with autologous fibroblasts, a cell capable of enhancing T cell survival (27, 28). T cells readily adhered to autologous HIF monolayers, and this close interaction resulted in a significantly ($p < 0.01$) greater viability compared with that of T cells cultured alone (Fig. 2). The HIF protective effect was evident in the absence of IL-2, but also in the presence of this cytokine (20 U/ml) ($p < 0.05$). A similar effect was also observed in cocultures, where T cells and HIF were physically separated from one another by a permeable membrane (Transwell system) ($p < 0.01$ in the absence of IL-2, and $p < 0.05$

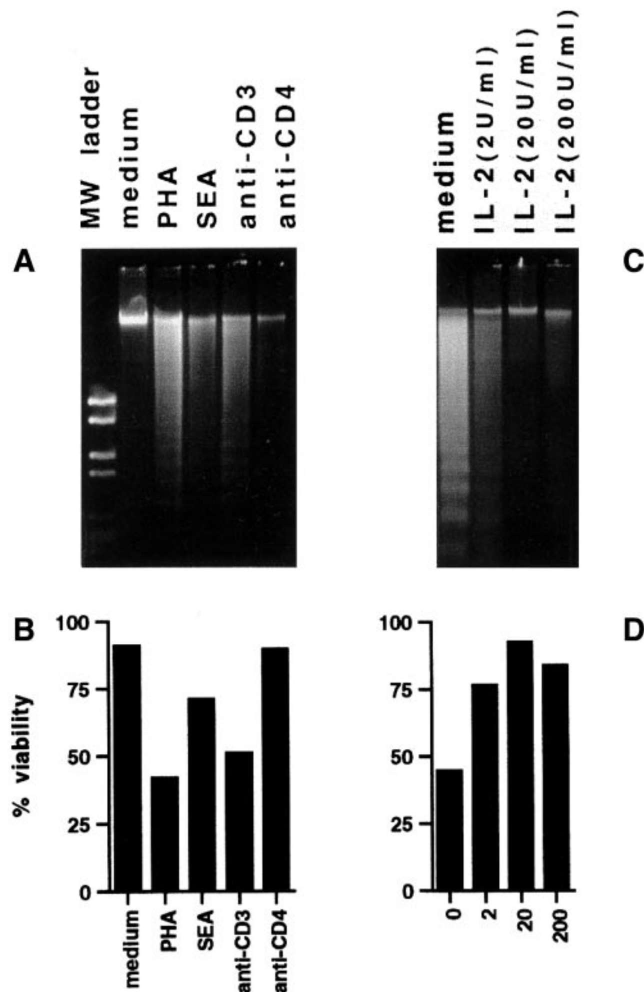

FIGURE 1. Effect of various stimuli on apoptosis of mucosal T cells. T cell lines were established from LPMC by stimulation with PHA and PMA followed by expansion with rIL-2. After 2–3 wk, T cells were exposed to PHA, SEA, and anti-CD3 or anti-CD4 mAbs for 18 h to induce apoptosis (*A* and *B*). Alternatively, the same cells were washed to remove IL-2 and cultured in medium alone or re-exposed to variable doses of IL-2 for 7 days (*C* and *D*). Viability was determined by trypan blue dye exclusion test (*B* and *D*), and extracted DNA was analyzed by agarose gel electrophoresis (*A* and *C*). Panels represent results obtained from parallel cultures. The figure is representative of six experiments, each performed with a T cell line from six different biopsies or LPMC.

in the presence of IL-2) (Fig. 2). The same results were obtained when autologous mucosal T cells and HIF from inflammatory bowel disease patients were used (data not shown).

Effect of FCM on mucosal T cell survival

The observation that viability of T cells increased in the absence of direct physical contact with HIF suggested that intestinal fibroblasts release soluble factors promoting mucosal T cell survival. To confirm this possibility, the IL-2-dependent CTLL-N cell line was exposed to different concentrations of FCM derived from supernatants of unstimulated HIF cultures. FCM consistently enhanced the viability of IL-2-starved CTLL-N cells in a concentration-dependent manner (Fig. 3). The enhancement was inversely proportional to and directly correlated with the degree of inhibition of apoptosis, both changes being of comparable magnitude. The antiapoptotic effect of FCM was confirmed by its ability to prevent DNA fragmentation of CTLL-N cells upon IL-2 removal (Fig. 4).

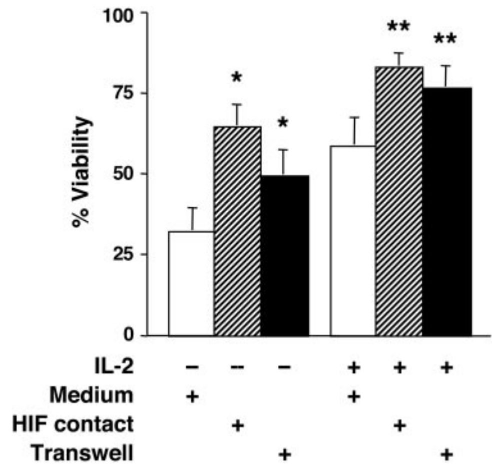

FIGURE 2. Protective effect of HIF on viability of autologous mucosal T cells. T cells obtained as described in Fig. 1 were cultured in medium alone, in direct contact with HIF (HIF contact), or separated by a porous membrane (Transwell), both in the absence (−) or presence (+) of IL-2. After 7 days, T cells viability was assessed by trypan blue dye exclusion test. Number of experiments: 17, each performed with a T cell line from 17 different LPMC. *, $p < 0.01$ for HIF contact and Transwell compared with medium in cultures without IL-2; **, $p < 0.05$ for HIF contact and Transwell compared with medium in cultures with IL-2.

Similar results were obtained with LPMC-derived T cell lines (Table I). The protective effect of FCM was not restricted to any particular T cell subset because the relative proportions of $CD4^+$, $CD8^+$, and $CD25^+$ cells were similar in cultures without or with FCM. In the IL-2 deprivation assay, $CD4^+$ cells were 82.6 ± 6.3 vs 79.2 ± 4.6, $CD8^+$ cells 16.9 ± 5.5 vs 20.3 ± 4.4, and $CD25^+$ cells 79.9 ± 2.3 vs 78.8 ± 2.7, respectively. In the Fas ligation assay, $CD4^+$ cells were 74.8 ± 3.0 vs 78.7 ± 4.8, $CD8^+$ cells 16.0 ± 6.9 vs 20.1 ± 5.0, and $CD25^+$ cells 71.9 ± 0.7 vs 67.9 ± 3.2, respectively. These results are based on four different T cell lines and five different FCM. The protective effect on T cell apoptosis was lost if FCM was heat-inactivated, and no effect was observed using medium not exposed to HIF (data not shown).

Investigation of antiapoptosis mediators in FCM

Having demonstrated the beneficial effect of FCM on mucosal T cell survival, we investigated which soluble mediators might be responsible for its antiapoptotic effect. We first tested the effect of recombinant or purified molecules reported to have T cell antiapoptotic activity, including IL-10 (17), IFN-β (29), and PGE_2 (30). Each of these molecules was initially tested for its potential protective effect against mucosal T cell apoptosis induced by both IL-2 deprivation and Fas ligation. The apoptotic index of IL-2-deprived T cells was significantly reduced by IL-10 and IFN-β ($p < 0.01$ for both), but not PGE_2 (Fig. 5). When apoptosis was induced by the CH11 Ab the same modulatory pattern was observed, with IL-10 and IFN-β, but not PGE_2, significantly ($p < 0.01$ for both) decreasing cell death (Fig. 5).

Next, we investigated whether these three mediators were actually present in the FCM, by measuring their levels by ELISA in supernatants of unstimulated or IL-1β- or IFN-γ-stimulated HIF. HIF spontaneously secreted both IL-10 and PGE_2 (Fig. 6, *A* and *B*). In contrast, although its mRNA was detectable by RT-PCR (data not shown), IFN-β protein was not found in any of the FCM tested, even when HIF cell concentration was increased and the culture time was extended up to 72 h. After stimulation with IL-1β, but not IFN-γ, the same HIF produced significantly increased

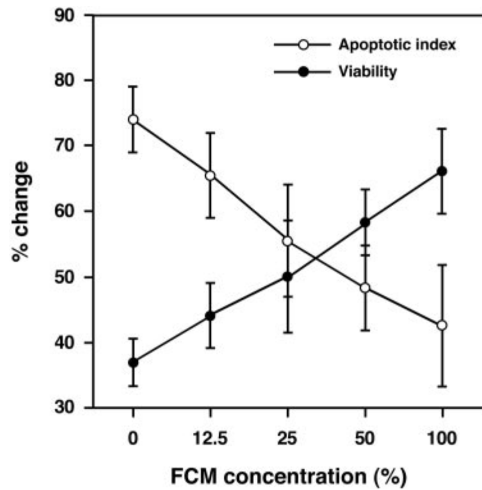

FIGURE 3. Effect of intestinal FCM on T cell viability and apoptosis. The IL-2-dependent CTLL-N cell line was washed to remove IL-2 and cultured in medium alone (0%) or increasing concentrations of FCM for 24 h. T cell viability was determined by trypan blue exclusion test, and apoptosis was measured by the acridine orange/ethidium bromide dye mix staining method. Number of experiments: three, each performed with FCM from three different HIF monolayers.

amounts of IL-10 and PGE_2 (both at $p < 0.01$) (Fig. 6, *A* and *B*), whereas IFN-β continued to be undetectable in the FCM. In addition, IL-2 and IL-15, two cytokines known to protect T cells from apoptosis (31), were also examined in the same FCM, but neither was detected.

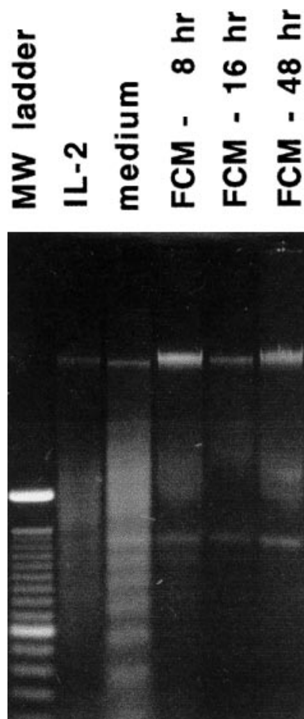

FIGURE 4. Effect of FCM on T cell apoptosis. Conditioned medium was obtained from supernatants of confluent HIF monolayers cultured for 8–48 h. The IL-2-dependent CTLL-N cell line was washed to remove IL-2 and then cultured in medium alone, IL-2 (20 U/ml), or FCM. After 24 h, T cell DNA was extracted and analyzed by agarose gel electrophoresis. The figure is representative of four separate experiments, each performed with FCM from four different HIF monolayers.

Table I. *Effect of intestinal FCM on T cell apoptosis*[a]

	Mucosal T Cells		CTLL-N Cells	
FCM	−	+	−	+
Apoptotic index (%)	47 ± 2	29 ± 8*	80 ± 6	43 ± 12*

[a] FCM was obtained from supernatants of confluent fibroblast monolayers. Mucosal T cells were derived from LPMC cultured with IL-2 (20 U/ml), and the CTLL-N cell line was continuously grown in IL-2. Cells were washed to remove IL-2 and then cultured for 24–48 h in medium alone or FCM. Apoptosis was measured by the acridine orange/ethidium bromide dye mix staining method. Number of experiments: mucosal T cell lines, 11; CTLL-N cells, 15. * $p < 0.01$ compared to medium alone.

IL-10 expression by HIF

The results so far indicated that IL-10 could mediate potent mucosal T cell antiapoptotic effect and was present in the FCM. In contrast, IFN-β, although effective, was not detected in FCM, whereas PGE_2, although present in FCM, was ineffective in preventing T cell apoptosis. Therefore, we focused our investigation on the characterization of IL-10 gene expression and protein secretion by HIF. Using Southern blot analysis of RT-PCR-amplified HIF RNA, the constitutive expression of IL-10 transcripts by HIF was demonstrated (Fig. 7). The baseline expression was up-regulated by proinflammatory mediators, such as IL-1β, as shown by the significant ($p < 0.01$) increase of IL-10 mRNA in HIF by in situ hybridization (Fig. 8). In addition, proteins contained in the FCM were extracted and submitted to microcapillary HPLC to identify IL-10 in the protein profile. Four main peaks were identified (A, B, C, and D), and peak B matched the one generated by rIL-10 (Fig. 9). Flow cytometric analysis did not detect the presence of IL-10 on the surface of unstimulated or IL-1β-stimulated HIF (data not shown).

Effect of HIF-derived IL-10 blockade on mucosal T cell apoptosis

To confirm that HIF-derived IL-10 was indeed the major antiapoptotic factor for mucosal T cells, its biological activity was blocked

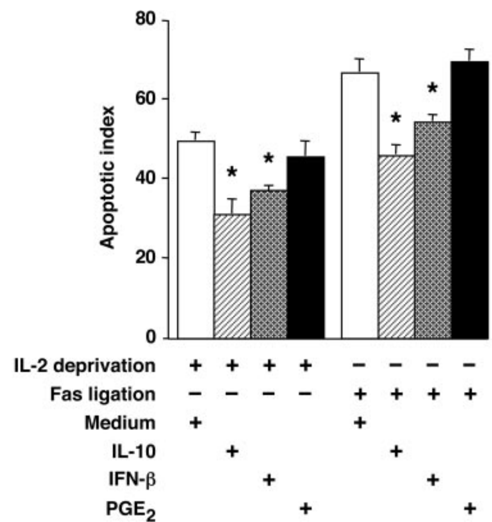

FIGURE 5. Effect of different soluble factors on mucosal T cell apoptosis. T cells obtained as described in Fig. 1 were cultured with and without human rIL-10 (10 ng/ml), IFN-β (10 ng/ml), and PGE_2 (50 pg/ml) for 24 h for IL-2 deprivation-induced apoptosis, and for 8 h for Fas ligation-mediated apoptosis. Number of experiments: six, each performed with a T cell line from six different biopsies or LPMC. *, $p < 0.01$ for IL-10- and IFN-β-treated compared with untreated cells for both IL-2-deprivation- and Fas ligation-mediated apoptosis.

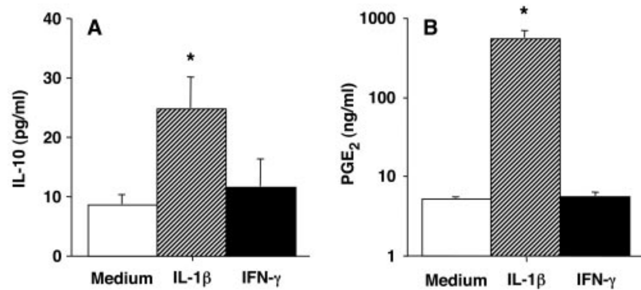

FIGURE 6. Spontaneous and cytokine-stimulated production of IL-10 and PGE_2 by HIF. HIF monolayers were cultured alone or in the presence of IL-1β (10 ng/ml) or IFN-γ (500 U/ml) for 24 h, and then the supernatants were collected and assayed for IL-10 (*A*) and PGE_2 (*B*) content by ELISA. Number of experiments: 10, each performed with 10 different HIF monolayers. $*$, $p < 0.01$ for IL-1β-stimulated compared with unstimulated cultures.

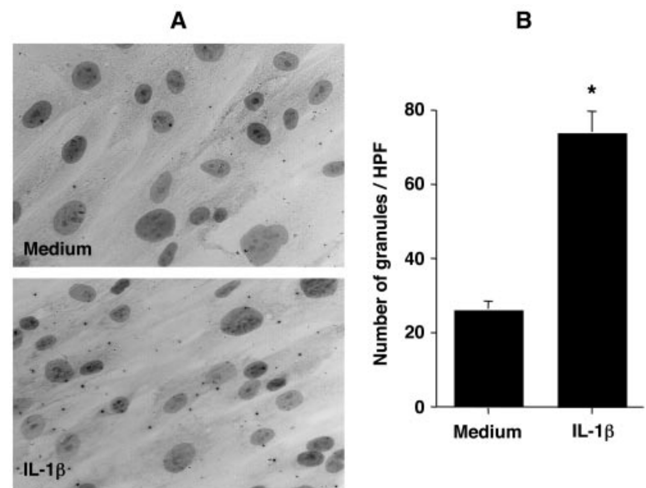

FIGURE 8. In situ hybridization for IL-10 mRNA in HIF. *A*, HIF monolayers were left unstimulated (Medium) or cultured in the presence of IL-1β (10 ng/ml) for 24 h, and then hybridized with an IL-10 RNA-specific probe. *B*, The number of granules present in five random high-power fields of unstimulated and IL-1β-stimulated monolayers. Number of experiments: three, each performed with three different HIF monolayers. $*$, $p < 0.01$ for IL-1β-stimulated compared with unstimulated cultures.

using neutralizing Abs or antisense oligonucleotides. First, using IL-2 deprivation to induce apoptosis, IL-10 activity was blocked by adding specific neutralizing Abs to FCM. This resulted in a significant ($p < 0.01$) increase in T cell apoptosis compared with the effect of FCM treated with isotype control Ab (Fig. 10). In the same experiments, neutralization of IFN-β or IFN-α failed to inhibit the antiapoptotic effect of FCM. When apoptosis was induced by activation of the Fas pathways, essentially the same results were observed ($p < 0.01$) (Fig. 10).

Second, each fraction obtained of HPLC-analyzed FCM was tested for its antiapoptotic activity using the same assay systems. As shown in Fig. 11, only peak B significantly ($p < 0.02$) inhibited the T cell apoptosis induced by both IL-2 deprivation and Fas ligation, and to a degree comparable to that of unfractionated FCM. Because these results indicated that the antiapoptotic activity of FCM was contained in fraction B, and previous results had indicated that IL-10 was primarily responsible for such activity, IL-10-neutralizing Abs were added to this fraction and tested it in the IL-2 deprivation assay. This resulted in the complete elimination of the antiapoptotic activity of fraction B, whereas the isotype control Ab had no effect (Fig. 12).

Finally, to further confirm that IL-10 was the key factor responsible for the antiapoptotic activity of FCM, HIF were transiently transfected with IL-10 antisense oligonucleotides, resulting in the specific reduction of both IL-10 mRNA and protein expression (data not shown). Transfected HIF were then cultured to generate FCM to be tested in the IL-2-deprivation- and Fas-mediated apoptosis. FCM generated from scrambled oligonucleotide-transfected HIF showed the same apoptosis inhibitory capacity as seen in previous experiments (Fig. 13). In contrast, FCM derived from HIF transfected with IL-10 antisense oligonucleotides showed no antiapoptotic effect, similar to that of medium alone (Fig. 13). When HIF were transfected with IFN-β antisense oligonucleotides, the resulting FCM showed the same antiapoptotic effect of FCM derived from cultures of HIF transfected with scrambled oligonucleotide (Fig. 13).

Proliferation

T cell proliferation and apoptosis are two intimately linked events (32). Therefore, we investigated whether the antiapoptotic effect of FCM was due solely to an inhibitory effect on cell death or also

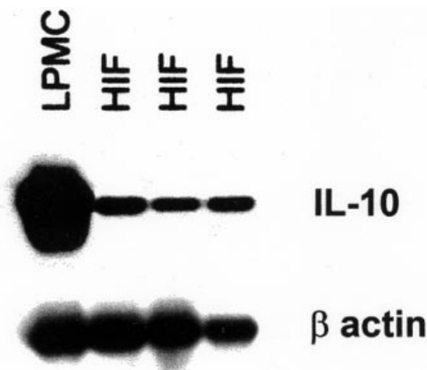

FIGURE 7. Constitutive expression of IL-10 mRNA by HIF. Total RNA was extracted from freshly isolated LPMC and three separate HIF monolayers, reverse-transcribed into cDNA using oligo(dT) primers, amplified by PCR, and analyzed by Southern blotting. The figure is representative of five separate experiments, each performed with five different LPMC and HIF extracts.

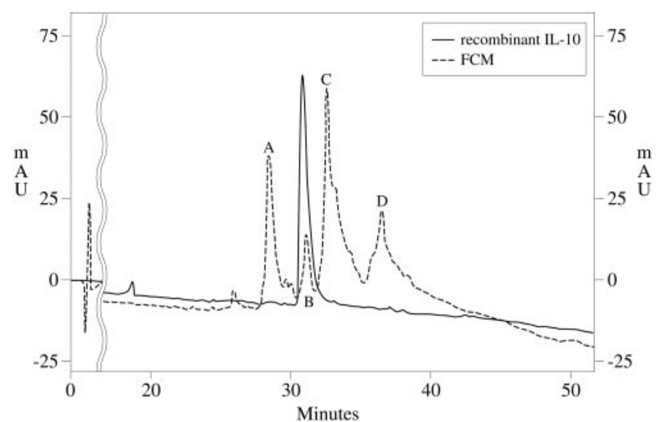

FIGURE 9. Reverse-phase capillary HPLC analysis of proteins extracted from FCM and rIL-10 protein. Four major peaks (A, B, C, and D) were detected in the FCM, with peak B overlapping with the peak induced by IL-10. The figure is representative of eight separate experiments, each performed with FCM from eight different HIF monolayers.

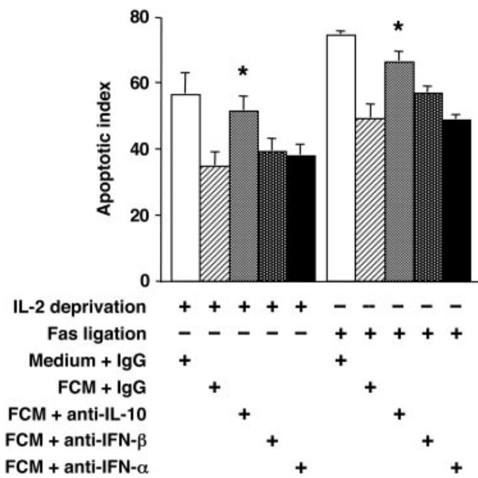

FIGURE 10. Effect of cytokine neutralizing Abs on the antiapoptotic effect of FCM on mucosal T cells. T cells obtained as described in Fig. 1 were cultured in medium containing IL-10 and IFN-$\beta\alpha$ isotype control Ab (rabbit IgG), FCM pretreated with IL-10 and IFN-β-α isotype control Ab (rabbit IgG), IL-10 Ab, IFN-β, or IFN-α Ab, for 24 h for IL-2 deprivation-induced apoptosis, and for 8 h for Fas ligation-mediated apoptosis. Number of experiments: seven, each performed with a T cell line from seven different biopsies or LPMC and FCM from seven different HIF monolayers. *, $p < 0.01$ for IL-10 Ab-treated compared with control Ab-treated FCM for both IL-2-deprivation- and Fas ligation-mediated apoptosis.

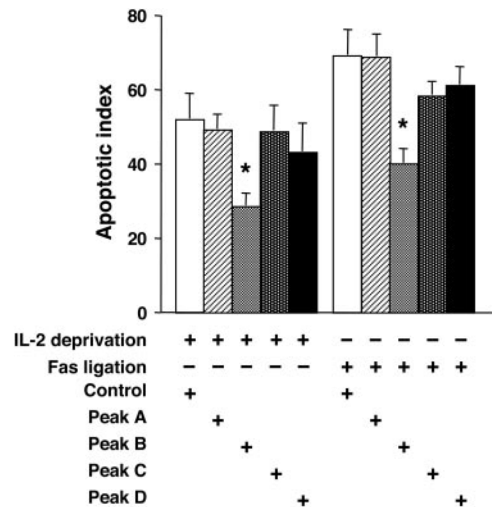

FIGURE 11. Effect of individual FCM-derived HPLC fractions on mucosal T cell apoptosis. T cells obtained as described in Fig. 1 were cultured in medium containing HPLC solvent (Control) or individual fractions (Peak A, B, C, and D) for 24 h for IL-2 deprivation-induced apoptosis, and for 8 h for Fas ligation-mediated apoptosis. Number of experiments: six, each performed with a T cell line from six different biopsies or LPMC and FCM from six different HIF monolayers. *, $p < 0.01$ for peak B compared with control or fraction A, C, or D, for both IL-2-deprivation- and Fas ligation-mediated apoptosis.

stimulated T cell proliferation. Mucosal T cells were exposed to medium alone, various concentrations of rIL-2, IL-10, and IFN-β as well as FCM from HIF transfected with scrambled oligonucleotides, IL-10, or IFN-β antisense oligonucleotides, and their proliferation was measured after 48 h. Except for IL-2, none of the other cytokines or FCM enhanced T cell proliferation above control levels (Fig. 14).

Discussion

This study provides evidence that intestinal mesenchymal cells exert a fundamental regulatory activity on acquired intestinal immunity by promoting the survival of mucosal T cells. This is accomplished by inhibition of apoptosis induced by lack of essential growth factors or Fas ligation, a protective effect mediated by two complementary pathways: one depending on the release of fibroblast-derived IL-10 and another on direct cell contact.

Intestinal mucosal T cells express high levels of Fas Ag, which makes them more susceptible to Fas-mediated apoptosis than peripheral blood T cells (33). This increased susceptibility to Fas-mediated apoptosis is also evident when they are stimulated through the CD2 pathway (34), suggesting that intestinal T cells may exhibit a unique response in regard to signals mediating survival and death. In contrast, our results show that mitogens, superantigens, and ligation of the CD3, but not CD4, receptor, as well as lack of IL-2 induce apoptosis of mucosal T cells. Thus, intestinal T cells also share with mature peripheral T cells some of the same activation- and growth factor deprivation-triggered death pathways (26, 35, 36).

Because of previous Ag exposure, mucosal T cells are almost exclusively CD45RO^{+} cells (37), and their long-term persistence throughout the intestine is essential to local immune homeostasis because they can recognize and react to the originally sensitizing dietary Ags, enteric flora Ags, and xenobiotics. The survival of mucosal T cells is probably mediated by a variety of cells and products, but we focused this study on the contribution of local fibroblasts because of the known ability of mesenchymal cells from several tissues, such as the synovium, lung, skin, gum, eye, and intestine, to prolong the life span of different types of leukocytes, including T cells, B cells, neutrophils, monocytes, and mast cells (27, 28, 38–43). In addition, intestinal fibroblasts are abundant, exist in intimate physical and functional contact with immune cells, and can produce a broad array of immunomodulatory factors (12, 44–47).

Some of the HIF-derived factors are clearly able to support mucosal T cell viability, as demonstrated by our observation that FCM enhanced viability of autologous mucosal T cells and markedly inhibited apoptosis-associated DNA fragmentation. These effects were not restricted to autologous T cells, because the same FCM could also enhance viability and inhibit apoptosis of mouse CTLL-N cells, indicating biological activity across species, as is often the case when human cytokines are used in murine systems. In addition, FCM antiapoptotic effect was not restricted to specific T cell subsets because CD4^{+}, CD8^{+}, and CD25^{+} cells were equally protected. Cytokines known to inhibit T cell apoptosis include those that signal through the common γ-chain of the IL-2 receptor, like IL-2, IL-4, IL-7, IL-9, and IL-15 (31, 48, 49), type I IFNs (IFN-α and -β) (48), and PGE_2 (30, 50). IL-10 also inhibits T cell apoptosis (35, 51), and we previously demonstrated the ability of this cytokine to block mucosal T cell apoptosis induced by a variety of signals, such as growth factor deprivation, Fas ligation, and NO (17). Several of the above cytokines are produced by HIF and could be responsible for the antiapoptotic effect of FCM.

Testing of recombinant or purified IL-10, IFN-β, and PGE_2 on mucosal T cells after IL-2 starvation or Fas ligation confirmed the antiapoptotic effect of IL-10 and IFN-β, but not PGE_2, suggesting that the antiapoptotic property of PGE_2 is not universal and may depend on the cell type it is acting upon. In fact, PGE_2 inhibits apoptosis of immature human thymocytes (50), T cell hybridomas (52), and activated murine splenocytes (30), but induces apoptosis in unstimulated thymocytes and resting peripheral lymphocytes (53, 54). Therefore, although intestinal mesenchymal cells produce abundant PGE_2 both spontaneously and after activation (47), its

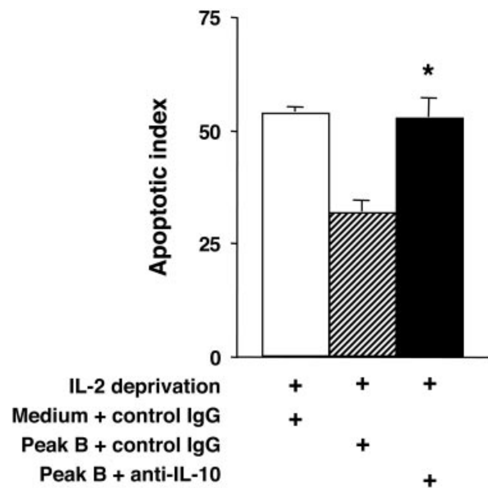

FIGURE 12. Effect of IL-10-neutralizing Ab treatment of peak B on mucosal T cell apoptosis. T cells obtained as described in Fig. 1 were cultured in medium containing IL-10 isotype control Ab (rabbit IgG), peak B containing IL-10 isotype control Ab (rabbit IgG), and IL-10 Ab for 24 h for IL-2 deprivation-induced apoptosis. Number of experiments: four, each performed with a T cell line from four different biopsies or LPMC and peak B from four different FCM-derived HPLC fractions. *, $p < 0.01$ for IL-10 Ab-treated compared with isotype control Ab-treated peak B.

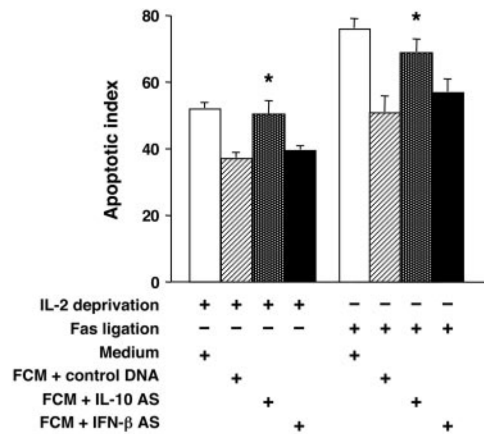

FIGURE 13. Effect of FCM derived from antisense (AS) oligonucleotide-transfected HIF on mucosal T cell apoptosis. T cells obtained as described in Fig. 1 were cultured in medium alone, FCM from scrambled antisense (control DNA) oligonucleotide-treated HIF, FCM from IL-10 antisense oligonucleotide-transfected HIF, or FCM from IFN-β antisense oligonucleotide-transfected HIF for 24 h for IL-2 deprivation-induced apoptosis, and for 8 h for Fas ligation-mediated apoptosis. Number of experiments: five, each performed with a T cell line from five different biopsies or LPMC and FCM from five different HIF monolayers. *, $p < 0.01$ for FCM from IL-10 antisense oligonucleotide-treated HIF compared with FCM from scrambled antisense oligonucleotide- or IFN-β antisense oligonucleotide-transfected HIF, for both IL-2-deprivation- and Fas ligation-mediated apoptosis.

contribution to maintenance of a hyporesponsive intestinal immune state may be through mechanisms other than modulation of cell death.

Having excluded PGE_2 as a major antiapoptotic factor in FCM, we next investigated IFN-β because this cytokine has been shown to be involved in the rescue of T cell apoptosis in various experimental and clinical systems (29, 48, 55, 56). Although IFN-β mRNA was present in HIF extracts, the protein was not detected in cultures of HIF unstimulated or HIF activated by IL-1β, whereas both IL-10 and PGE_2 were detected and significantly up-regulated in the same cultures. This was a surprising finding in view of reports based on blocking experiments and immunohistochemical staining, although not direct protein measurement, claiming that IFN-β is the main factor responsible for the antiapoptotic effect on T cells of medium conditioned by fibroblast from synovium, skin, lung, and conjunctiva (29, 57, 58). In contrast, the absence of biologically active IFN-β in HIF FCM was confirmed by the inability of IFN-β-specific Abs to block the protective effect of FCM against mucosal T cell apoptosis caused by both IL-2 deprivation and Fas ligation. The same was true for IFN-α. In addition, the fact that IFN-β was not the biologically active antiapoptotic moiety in FCM was further confirmed by the persistence of the protective effect, in both the IL-2 deprivation and Fas ligation systems, of FCM derived after HIF had been transfected with IFN-β antisense oligonucleotides. Taken together, these results suggest that the intestinal mucosal microenvironment differs from other tissues in the body, not only in regard to its intrinsic ability to maintain an overall immunosuppressive tone, but also the unique mechanisms to foster the survival of Ag-primed mature mucosal T cells in a PGE_2- and IFN-β-independent manner.

To definitively prove that IL-10 was the main antiapoptotic mediator of HIF supernatants, as suggested by the expression of IL-10 mRNA by HIF, detection of immunoreactive IL-10 in HIF FCM, and the IL-10-neutralizing Ab blocking experiments, HPLC was used to isolate this cytokine directly in the FCM. Peak B of HIF-conditioned medium overlapped with the peak of rIL-10, was the only one capable to significantly inhibit mucosal T cell apoptosis induced by IL-2 starvation or Fas-ligation and, when anti-IL-10 Ab was added, its antiapoptotic effect was eliminated. The sum of these results leads to the conclusion that IL-10 is indeed the active antiapoptotic principle present in HIF FCM, a conclusion corroborated by the selective elimination of the protective effect in supernatants derived from HIF transfected with IL-10 antisense oligonucleotides. Mesenchymal cells from different tissues produce a variety of proinflammatory and immunoregulatory cytokines, and IL-10 protein is found in synoviocytes cultures (59, 60), and IL-10 mRNA in human duodenal fibroblasts (44). HIF also produce multiple cytokines and chemokines (12, 45, 46), but this study is the first to describe the production of bioactive IL-10 protein by intestinal mesenchymal cells and demonstrate its role as an antiapoptotic factor for autologous mucosal T cells. The amounts of IL-10 were relatively low in FCM, but because fibroblasts are abundant and live in intimate physical proximity of T cells, its antiapoptotic effect is probably exerted through a paracrine action in vivo.

Finally, we investigated whether the antiapoptotic effect of FCM was associated with induction of mucosal T cell proliferation. In contrast to IL-2, but similarly to rIL-10 and IFN-β, FCM from untransfected HIF, and FCM from HIF transfected with scrambled DNA, IL-10 or IFN-β antisense oligonucleotides failed to induce mucosal T cell proliferation. This suggests that HIF-derived IL-10 maintains survival of mature T cell in a quiescent state like type I IFNs do (55, 56). This property is particularly important in the intestine because it may prevent T cell activation in the highly stimulatory environment of the intestinal mucosa.

The timely death of clonally expanded Ag-specific T cells in the resolving phase of an immune response is essential to immune homeostasis (61). When this fails to occur, there is an excessive accumulation of T cells and development of chronic inflammation (62) and, for this reason, induction of apoptosis is under consideration as a new therapeutic tool in autoimmune diseases (63). Resistance of tissue T cell to apoptosis has been described in various conditions like rheumatoid arthritis, cutaneous inflammation, and asthma (57, 64, 65), and also in the intestine involved by

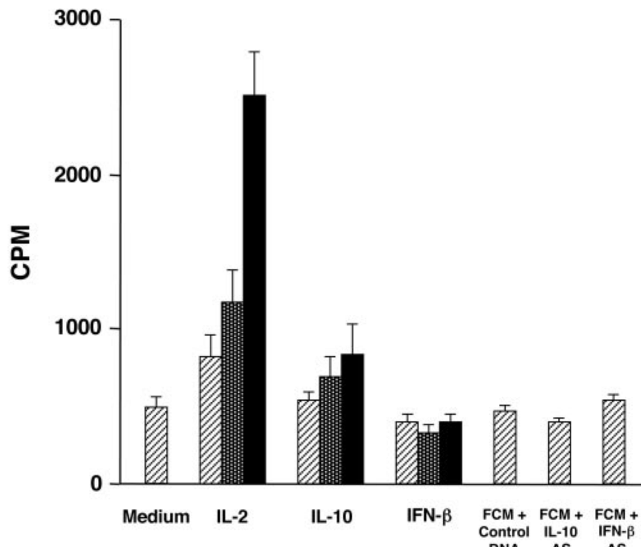

FIGURE 14. Effect of antiapoptotic cytokines and FCM derived from antisense (AS) oligonucleotide-treated HIF on mucosal T cell proliferation. T cells were cultured in rIL-2 (at 0.1, 1, and 10 U/ml; hatched, cross-hatched, and black bars, respectively), IL-10, IFN-β (both at 0.1, 1, and 10 ng/ml; hatched, crosshatched and black bars, respectively), FCM from scrambled antisense oligonucleotide-treated HIF, FCM from IL-10 antisense oligonucleotide-treated HIF32, or FCM from IFN-β antisense oligonucleotide-treated HIF for 48 h, and proliferation was measured by tritiated thymidine uptake. Number of experiments: three, each performed with a T cell line from three different biopsies or LPMC and FCM from three different HIF monolayers.

Crohn's disease (17, 66). The latter condition is of particular interest because induction of mucosal T cell apoptosis has been proposed as a key mechanism for the clinical efficacy of TNF-α-targeting therapies (67, 68). In contrast, rIL-10 has no significant beneficial effect in Crohn's disease patients (69, 70) and, in light of our results, it is tempting to speculate that this is due to promoting the survival of infiltrating inflammatory cells. Therefore, the seemingly beneficial effect of HIF-derived IL-10 in preserving viability of memory T cells in the normal intestinal mucosa may become detrimental under inflammatory conditions and contribute to disease chronicity.

In conclusion, in contrast to other organs and systems where survival of memory cells is mediated by TCR activation, type I IFNs, or PGE_2, in the intestine this is achieved through the antiapoptotic effects of local fibroblast-derived IL-10. This role of IL-10 may be selective to the intestinal mucosa, where this cytokine also plays a critical role in generation of regulatory cells and maintenance of immune homeostasis (71, 72). These results underscore the crucial importance of studying tissue-specific cells and products to understand the true mechanisms of organ homeostasis in unique microenvironments like the intestinal mucosa.

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Disclosures

The authors have no financial conflict of interest.

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