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Human γδ-T Lymphocytes Express and Synthesize Connective Tissue Growth Factor: Effect of IL-15 and TGF-β1 and Comparison with αβ-T Lymphocytes[1]

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T lymphocytes bearing the γδ-TCR accumulate during wound healing and inflammation. However, the role of γδ-T lymphocytes in fibrogenic tissue reactions is not well understood. Therefore, we addressed the question of whether human γδ-T cells express and synthesize connective tissue growth factor (CTGF), a factor known to regulate fibrogenesis and wound healing. In addition, the lymphoblastic leukemia T cell line (Loucy) that possesses characteristics typical of γδ-T cells was used as a model to evaluate the regulation of CTGF gene expression. Blood γδ-T cells isolated from healthy donors were grown in the presence of IL-15/TGF-β1 for 48 h and assessed for the expression and synthesis of CTGF. Nonstimulated human blood γδ-T cells and Loucy γδ-T cells expressed low levels of CTGF mRNA. Costimulation of the cells with IL-15 and TGF-β1 resulted in a substantially increased level of CTGF mRNA expression within 4–8 h, and it remained elevated for at least 48 h. In contrast, no CTGF mRNA was detected when nonstimulated and stimulated human CD4$^+$ αβ-T cells were analyzed. In addition, Western blot analysis of human γδ-T cell lysates prepared 4 days following stimulation with IL-15 and TGF-β1 revealed a 38-kDa CTGF protein in cell lysates of human γδ-T cells. Detection was confirmed using Colo 849 fibroblasts, which can constitutively express high levels of CTGF. In conclusion, we herein present novel evidence that in contrast to CD4$^+$ αβ-T cells human γδ-T cells are capable of expressing CTGF mRNA and synthesizing its corresponding protein, which supports the concept that γδ-T cells may contribute to wound healing or tissue fibrotic processes. ***The Journal of Immunology,*** **2003, 170: 153–157.**

Connective tissue growth factor (CTGF)[3] is a cysteine-rich, heparin-binding peptide, originally identified as a growth factor secreted by HUVEC (1). CTGF is also produced by fibroblasts and muscle cells, but not by human lymphocytes (2). Functionally, the growth factor has been shown to regulate cell adhesion, migration, and proliferation of mesenchymal cells (3). In addition, CTGF acts as a mitogenic stimulus on fibroblasts and induces the expression of the extracellular matrix molecules collagen type I (α_1 chain), fibronectin, and integrin α_5 in vitro (4). CTGF mRNA expression has also been found to be associated with areas of tissue fibrosis in systemic sclerosis (5), in other fibrotic disorders (6, 7), and in granulation tissue beds following tissue injury (2, 7), suggesting a role for the growth factor in fibrotic connective tissue formation and during wound healing.

T lymphocytes bearing γδ-TCR represent a small subpopulation of human peripheral lymphocytes. They differ from αβ-T cells in several parameters, including ontogenic appearance, tissue distribution, and Ag recognition (8). Although increased numbers of γδ-T lymphocytes have been observed in a number of infectious diseases (9, 10), the physiologic and pathophysisologic roles of the cells in these disorders are not fully understood. There is evidence showing that γδ-T cells accumulate during the fibrogenic inflammation underlying wound healing (11) and in human infectious disease lesions (9). In addition, γδ-T cells have been shown to secrete chemotactic factors involved in the recruitment of inflammatory cells (12, 13) as well as a number of cytokines (14). Further, γδ-T cells release factors such as keratinocyte growth factor (KGF; fibroblast growth factor-7) (15) that can affect epithelial growth and repair. However, the production of other fibroblast-stimulating factors by γδ-T cells has not been described. To gain further insight into the functional properties of γδ-T lymphocytes, we assessed their capacity of expressing and synthesizing CTGF and compared the results with a cloned γδ-T cell line (Loucy).

Materials and Methods

Isolation of CD4$^+$ αβ- and γδ-T cells

Peripheral blood was obtained from healthy donors, and the lymphocytes were separated by Ficoll density gradient centrifugation. γδ-T cells were purified using the MACS TCRγ/δ microbead kit (Miltenyi Biotec, Bergish Gladbach, Germany). γδ-T cells were positively selected using anti-γδ mAbs. CD4$^+$ αβ-T cells were also positively selected using the CD4$^+$ T cell isolation kit (Miltenyi Biotec). The γδ-T cell line (Loucy) and the fibroblast cell line (Colo 849) were obtained from the German collection of micro-organisms and cell cultures (DMSZ, Braunschweig, Germany).

All cells used in this study were grown in RPMI 1640 medium (Cell Concept, Umkirch, Germany) supplemented with 10% FCS (Life Technologies, Kalsruhe, Germany), 2 mM L-glutamine (Biochrom, Berlin, Germany), 100 IU/ml ampicillin, 100 ng/ml streptomycin, and 100 ng/ml gentamicin (Roche, Mannheim, Germany). The cells (1×10^6/well) were grown in the presence or the absence of IL-15 (10 ng/ml)/TGF-β1 (1 ng/ml; Cell Concept, Umkirch, Germany).

Total RNA isolation and RT-PCR

Total RNA was isolated at different time points (0, 4, 8, 24, and 48 h) from 1×10^6 cultured cells using the High Pure RNA isolation kit (Roche). For time zero, total RNA was isolated shortly after magnetic sorting from equal number of cells. First-strand cDNA was synthesized using the First Strand

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[3] Abbreviations used in this paper: CTGF, connective tissue growth factor; KGF, keratinocyte growth factor.

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RT-PCR Kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's protocol. One-tenth of the cDNA obtained was amplified in each PCR reaction using PerkinElmer AmpliTaq Gold polymerase (Roche) in a Gene Amp PCR System 9600 thermal cycler (PerkinElmer, Norwalk, CT). Forty cycles of amplification were conducted as follows: an initial heating step of 10 min at 95°C, followed by 94°C for 45 s, 61°C for 45 s, 72°C for 1 min, and a final synthesis of 10 min at 72°C. The same annealing temperature was used for both CTGF and β-actin. Specific primer sequences were designed from available GenBank sequences: CTGF sense primer, 5′-ACGGCGAGGTCATGAAGAAGAACA-3′; and CTGF antisense primer, 5′-TGGGGCTACAGGCAGGTCAGTG-3′. The amount of each cDNA was standardized using β-actin primers: β-actin sense primer, 5′-GGCGGGCATTCACGAGACCACCTA-3′; and β-actin antisense primer, 5′-GAGCCGCCGATCCACACCGAGTAT-3′.

The PCR products were separated by flat bed electrophoresis in 1.5% agarose gels (Roche), visualized employing ethidium bromide staining on a UV transilluminator, recorded using Phoretix Grabber software, and analyzed densitometrically using Phoretix 1D Advanced version 4.00 software (Biostep, Jahnsdorf, Germany).

SDS-PAGE and Western blot analysis

All cells used for Western blot were cultured in RPMI 1640 medium in the absence or the presence of IL-15 (10 ng/ml)/TGF-β1 (1 ng/ml). The cells (2×10^6) were harvested on day 4 by centrifugation at $350 \times g$ and were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 120 mM NaCl, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 1 μM PMSF, 10 μg/ml aprotinin, and 10 μM leupeptin; Sigma-Aldrich, Taufkirchen, Germany) by incubating them on ice for 30 min. The cell lysates were separated from cell debris by centrifugation at $14{,}000 \times g$, and the protein concentrations were determined using a spectrophotometer. The cell lysates (containing 20 μg of total protein) were diluted with nonreducing Tris-glycine-SDS sample buffer (Invitrogen, Frankfurt am Main, Germany), heated at 95°C for 5 min, and subjected to 12% SDS-PAGE gel. Transfer to nitrocellulose (Invitrogen) was performed by electroblotting. Equal loading of protein was verified by staining the membrane with Ponceau S (Sigma-Aldrich, Steinheim, Germany). Membranes were blocked in 1% blocking solution (Roche) in TBS (50 mM Tris-base (pH 7.5) and 150 mM NaCl) overnight at 4°C. The membranes were incubated for 1 h at room temperature with rabbit anti-human CTGF polyclonal Ab (Pab2; Fibrogen, San Francisco, CA) diluted 1/1000 in TBS/0.5% blocking solution. The anti-actin mAb (Chemicon International, Hofheim, Germany) was diluted 1/2000. After washing four times with TBS-T, blots were incubated for 1 h with HRP-conjugated anti-rabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected with 3,3′-diaminobenzidine substrate (Roche). The blots were recorded using Phoretix Grabber software and were analyzed densitometrically using Phoretix 1D Advanced version 4.00 software (Biostep).

Results

CTGF mRNA expression in human γδ-T cells

The first question addressed was whether human blood γδ-T cells express CTGF mRNA. γδ-T cells with a purity of >95% and a viability of >98% were used for the experiments.

In previous experiments we established that costimulation of the cells with IL-15 (10 ng/ml) and TGF-β1 (1 ng/ml) resulted in a maximal expression of the activation Ag $\alpha_E\beta_7$ (CD103) compared with each stimulus alone (16). Therefore, in all additional experiments cells were costimulated with both cytokines at the concentrations indicated.

As shown in Fig. 1, CTGF mRNA could be detected in freshly isolated human γδ-T lymphocytes at a low level. In the absence of the cytokines, the expression of CTGF increased slightly over a culture period of 48 h. When human blood γδ-T cells were exposed to IL-15/TGF-β1, the expression of CTGF mRNA increased

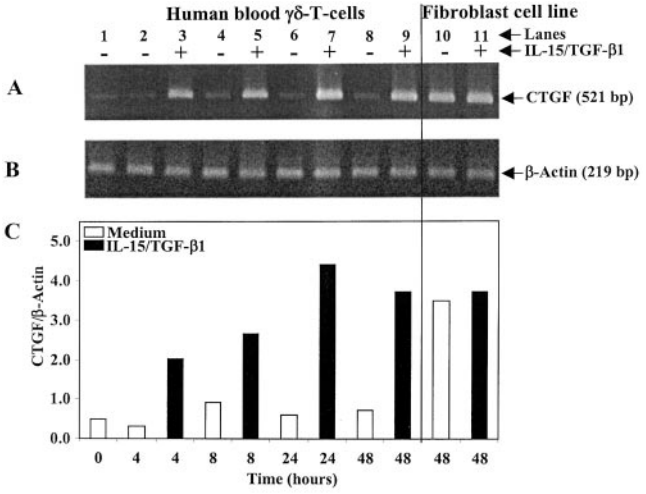

FIGURE 1. CTGF mRNA expression in human blood γδ-T cells. The lane numbers in *A* represent the corresponding lanes in *B* and the bar graphs in *C*. The time points indicated below the bar graph (*C*) also apply to the corresponding lanes shown in *A* and *B*. *A*, Freshly isolated human blood γδ-T cells (*lanes 1–9*) and fibroblast cells (*lanes 10* and *11*) were cultured in the absence (−) or in the presence (+) of TGF-β1 (1 ng/ml)/IL-15 (10 ng/ml). Since Colo 849 fibroblast cell line expresses CTGF constitutively (Fig. 4), one time point was selected (48 h) and used as a positive control. As described in *Materials and Methods*, the cells were harvested at different time points (0, 4, 8, 24, and 48 h), total RNA was isolated, and RT-PCR was performed on each sample. The PCR products of 521 and 219 bp represent CTGF and β-actin mRNA, respectively. A markedly elevated CTGF mRNA signal was achieved within 48 h after stimulation of human blood γδ-T cells with IL-15/TGF-β1 (*lanes 3*, *5*, *7*, and *9*). *B*, β-Actin demonstrated that equivalent quantities of RNA were used for each sample. *C*, The ordinate demonstrates the ratio of CTGF/β-actin PCR products. Data are representative of four experiments that gave similar results.

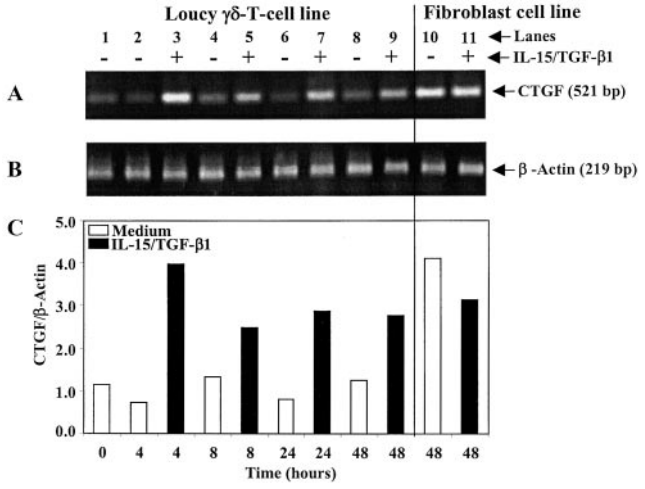

FIGURE 2. CTGF mRNA expression in Loucy γδ-T cell line. The lane numbers in *A* represent the corresponding lanes in *B* and the bar graphs in *C*. The time points indicated below the bar graph (*C*) also apply to the corresponding lanes shown in *A* and *B*. *A*, Loucy γδ-T cells (*lanes 1–9*) and fibroblast cells (*lanes 10* and *11*) were cultured in the absence (−) or the presence (+) of TGF-β1 (1 ng/ml)/IL-15 (10 ng/ml). Since the Colo 849 fibroblast cell line expresses CTGF constitutively (Fig. 4), one time point was selected (48 h) and used as a positive control. As described in *Materials and Methods*, the cells were harvested at different time points (0, 4, 8, 24, and 48 h), total RNA was isolated, and RT-PCR was performed on each sample. The PCR products of 521 and 219 bp represent CTGF and β-actin mRNA, respectively. An increased CTGF mRNA expression compared with nonstimulated cells was observed within 48 h after stimulation of Loucy γδ-T cells with IL-15/TGF-β1 (*lanes 3*, *5*, *7*, and *9*). *B*, β-Actin demonstrated that equivalent quantities of RNA were used for each sample. *C*, The ordinate demonstrates the ratio of CTGF/β-actin PCR products. Data are representative of four experiments that gave similar results.

at 4–8 h and was maintained for at least 48 h following stimulation of the cells. CTGF mRNA expression could also be detected in the human γδ-T cell line Loucy at a low level without stimulation (Fig. 2). As with the freshly purified blood lymphocytes, CTGF mRNA expression significantly increased when cells were cultured in the presence of IL-15/TGF-β1.

In contrast to human γδ- and Loucy T lymphocytes, neither nonstimulated nor cytokine-stimulated $CD4^+$ αβ-T lymphocytes expressed CTGF mRNA (Fig. 3) at any time point.

To confirm that CTGF mRNA was indeed detected in our assay system we also assessed the human melanoma fibroblast cell line (Colo 849) that could constitutively express CTGF (Fig. 4). As shown in Figs. 1–3 (*lanes 10* and *11*), Colo 849 fibroblast cells were used as a positive control for CTGF mRNA expression.

Detection of CTGF protein expression by Western blotting

Human γδ-T lymphocytes were assessed for the production of CTGF protein using the Western blot technique. As depicted in Fig. 5, very little of the 38-kDa CTGF protein could be detected in cell lysates of nonstimulated, freshly isolated blood γδ-T cells (Fig. 5, *lane 4*) and the Loucy γδ-T cell line (Fig. 5, *lane 6*) incubated for 96 h. However, in the presence of IL-15/TGF-β1, a notable increase in CTGF protein was observed in freshly isolated blood γδ-T lymphocytes (Fig. 5, *lane 5*). Although less pronounced, an increased CTGF protein signal could also be observed when stimulated Loucy γδ-T cells were assessed (Fig. 5, *lane 7*) compared with the control cells. In contrast, $CD4^+$ αβ-T cells did not express CTGF under any of experimental conditions (Fig. 5, *lanes 2* and *3*). When Colo 849 fibroblast cells were analyzed, CTGF protein was detected in nontreated cells that was not further increased in the presence of IL-15/TGF-β1 (Fig. 5, *lanes 8* and *9*).

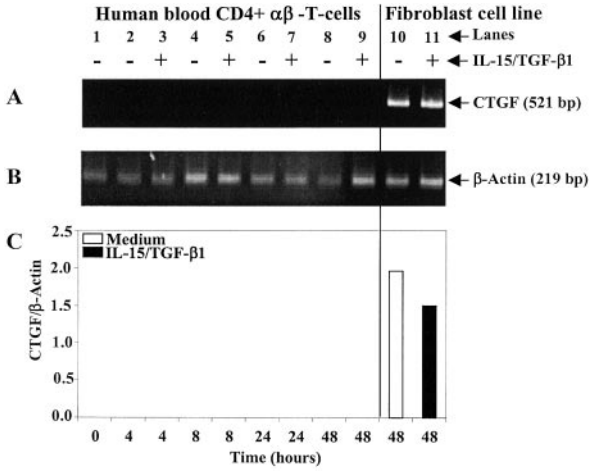

FIGURE 3. CTGF mRNA expression in human $CD4^+$ αβ-T cells. The lane numbers in *A* represent the corresponding lanes in *B* and the bar graphs in *C*. The time points indicated below the bar graph (*C*) also apply to the corresponding lanes shown in *A* and *B*. *A*, Freshly isolated human $CD4^+$ αβ-T cells (*lanes 1–9*) and Colo 849 fibroblast cell line (*lanes 10* and *11*) were cultured in the absence (−) or the presence (+) of TGF-β1 (1 ng/ml)/IL-15 (10 ng/ml). As described in *Materials and Methods*, the cells were harvested at different time points (0, 4, 8, 24, and 48 h), total RNA was isolated, and RT-PCR was performed on each sample. The PCR products of 521 and 219 bp represent CTGF and β-actin mRNA, respectively. $CD4^+$ αβ-T cells did not express CTGF mRNA at any time point tested. Since fibroblast cells express CTGF constitutively (Fig. 4), they were cultured for 48 h and used as a positive control. *B*, β-Actin demonstrated that equivalent quantities of RNA were used for each sample. *C*, The ordinate demonstrates the ratio of CTGF/β-actin PCR products. Data are representative of three experiments that produced similar results.

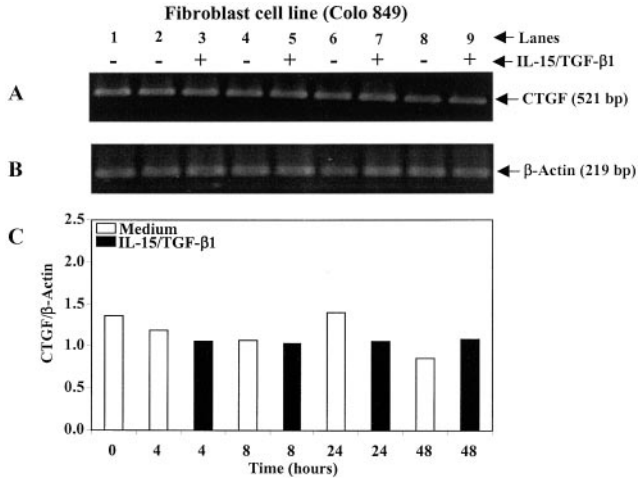

FIGURE 4. CTGF mRNA expression in the Colo 849 fibroblast cell line. The lane numbers in *A* represent the corresponding lanes in *B* and the bar graphs in *C*. The time points indicated below the bar graph (*C*) also apply to the corresponding lanes shown in *A* and *B*. *A*, Fibroblast cells (*lanes 1–9*) were cultured in the absence (−) or the presence (+) of TGF-β1 (1 ng/ml)/IL-15 (10 ng/ml). As described in *Materials and Methods*, the cells were harvested at different time points (0, 4, 8, 24, and 48 h), total RNA was isolated, and RT-PCR was performed on each sample. The PCR products of 521 and 219 bp represent CTGF and β-actin mRNA, respectively. CTGF could be expressed constitutively in both nonstimulated and stimulated fibroblast cells (Colo 849). *B*, β-Actin demonstrated that equivalent quantities of RNA were used for each sample. *C*, The ordinate demonstrates the ratio of CTGF/β-actin PCR products. Data are representative of three experiments that produced similar results.

Discussion

γδ intraepithelial lymphocytes have been shown to modulate local inflammatory fibrotic and infectious conditions (17, 18) and contribute to fibrogenesis and wound healing (11). However, since little is known about the potential signals linking γδ-T cells to fibrogenesis, the purpose of the present study was to determine whether human γδ-T cells express the fibrogenic cytokine CTGF. The data presented herein demonstrate for the first time that nonstimulated γδ-T cells express CTGF mRNA in vitro, which is significantly up-regulated in response to costimulation with IL-15 and TGF-β1 over a period of at least 48 h. The data also show that γδ-T cells produce CTGF protein, suggesting that the cells are able to secrete the cytokine. In contrast, $CD4^+$ αβ-T cells did not produce CTGF mRNA under present experimental conditions regardless of the presence or absence of IL-15/TGF-β1.

The results obtained with human blood γδ-T cells could be confirmed using the human γδ-T cell line (Loucy), a cloned cell line representing a Vγ9/Vδ2-subtype. It is interesting to note that Loucy cells show a response pattern comparable to that seen in human blood γδ-T lymphocytes. Although Loucy cells appear to be less susceptible to the induction of CTGF mRNA as well as CTGF protein expression by IL-15 and TGF-β1, our data suggest that they may be used as a model cell line for γδ-T lymphocytes in evaluating the role of CTGF and possibly other growth factors.

T cells that express αβ-TCRs comprise the vast majority of mature T cells in peripheral blood. In contrast, T cells that bear the γδ-TCR constitute a minor population of mature T cells in the circulation and lymphoid tissue, but are greatly increased during infection. αβ- and γδ-T cells share similarities in that both differentiate primarily in the thymus, possess common cell surface Ags, and have a diversity of clonotypic receptors associated with the CD3 complex. Despite these common properties, αβ- and γδ-T

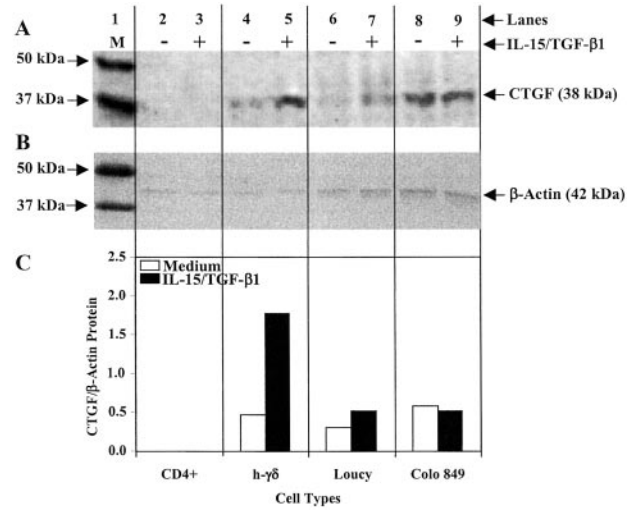

FIGURE 5. Detection of CTGF protein expression. The lane numbers in *A* represent the corresponding lanes in *B* and the bar graphs in *C*. *Lanes 2, 4, 6,* and *8* represent nonstimulated cells, whereas *lanes 3, 5, 7,* and *9* represent stimulated cells. *A,* Cell lysates used for Western blotting were extracted from 2×10^6 stimulated freshly isolated human $CD4^+$ αβ-T cells (*lanes 2* and *3*), freshly isolated human blood γδ-T cells (*lanes 4* and *5*), Loucy γδ-T cells (*lanes 6* and *7*), and Colo 849 fibroblast cells (*lanes 8* and *9*). All cell types were cultured in either the absence (−) or the presence (+) of TGF-β1 (1 ng/ml)/IL-15 (10 ng/ml) for 96 h. An increased level of CTGF protein (38 kDa) compared with the nonstimulated cells (*lanes 4* and *6*, respectively) was detected by freshly isolated human blood γδ-T cells (*lane 5*) and Loucy γδ-T cells (*lane 7*) after stimulation with IL-15/TGF-β1. Fibroblast cells synthesize CTGF protein constitutively (*lanes 8* and *9*). $CD4^+$ αβ-T cells did not synthesize CTGF protein (*lanes 2* and *3*). The arrows indicate the sizes of the proteins. *B,* β-Actin demonstrated that equivalent quantities of total protein were used for each sample. *C,* The ordinate demonstrates the ratio of CTGF/β-actin protein synthesis. Western blots are representative of four experiments that gave similar results. M, Prestained protein marker (*lane 1*); $CD4^+$, $CD4^+$ αβ-T cells; h-γδ, human γδ-T cells; Loucy, γδ-T cell line; Colo 849, fibroblast cell line.

cells also show significant differences. Unlike αβ-T cells, the majority of γδ-T cells lack the functional expression of CD4 and CD8 molecules. Moreover, the manner in which γδ-T cells recognize alloantigen appears to be different from that of αβ-T cells. The data presented herein indicate another potential difference between the T cell subpopulations with respect to the production of CTGF when stimulated in the presence of the IL-15/TGF-β1 employed in our experiments. Although the results do not rule out the possibility that αβ-T lymphocytes produce this factor under different experimental conditions, these observations further support the idea that both αβ- and γδ-T cells fulfill unique functional roles within the immune system.

CTGF is a member of the closely related connective tissue growth factor/Cyr61/NOV (CCN) family of cytokines that has been shown to function as a downstream mediator of TGF-β action on connective tissue cells, where it stimulates cell proliferation and extracellular matrix synthesis (3). Because the biological actions of TGF-β are complex and affect many different cell types, CTGF may serve as a more specific target for selective intervention in connective tissue formation during wound repair or fibrotic conditions. CTGF has been shown to be synthesized by the NRK fibroblast cell line after activation with TGF-β (2, 4), by dermal fibroblasts in association with repair processes (2), and by muscle and epithelial cells, but not in other cell types, such as leukocytes (19). Our results extend the list of potential CTGF-producing cells to include human γδ-T lymphocytes.

A number of studies suggest that γδ-T cells can protect the host from pathogenic and non-pathogenic insults (18). For example, we have previously shown that the presence of γδ-T cells reduces the extent of pulmonary inflammation and fibrosis induced by bleomycin (20). In addition, it has been demonstrated that the presence of γδ-T cells plays an essential role in the survival of host against intraepithelial pathogens such as *Nocardia* sp. (18). This protective role contrasts with the observation that γδ-T cells are capable of producing KGF as well as other cytokines preferentially associated with inflammatory processes. Our finding that γδ-T lymphocytes express CTGF mRNA and protein adds another cytokine to those produced by these cells. This protein has been shown to be involved in several fibrotic diseases and plays a critical role in fibrogenesis and tissue remodeling after injury.

The ability of γδ-T lymphocytes to secrete a factor that can affect epithelial growth and repair as well as factors that can mediate inflammation and fibrogenesis suggests a dual functionality of the cells. The reason for the discrepancy between a protective role and the production of pro-inflammatory or fibrogenic factors by γδ-T cells is not immediately apparent, but can be explained by the differential expansion of functionally different γδ-T cell subpopulations. Alternatively, a different response pattern of γδ-T cells may be determined in a specific inflammatory microenvironment. Another explanation may relate to the fact that epithelial repair processes require the formation of extracellular matrix proteins.

Although γδ-T lymphocytes tend to accumulate in association with inflammation, their expansion is not limited to the early stages of host response to infection or tissue injury. In murine influenza A infection, the number of γδ-T cells in the lung increased at a much higher rate after 7 days of viral infection (21). This finding suggests that the γδ-T cells in this disease are not only focused on the elimination of the virus-infected cells, but also on the repair processes of the host following a pathogen-inflicted injury. Since KGF-treated alveolar epithelial type II cells markedly increase epithelial repair (22), and KGF has been shown to prevent bleomycin-induced end-stage pulmonary injury (23), it is conceivable that KGF released by γδ-T cells may facilitate wound healing via restoration of the epithelial cell layer after lung injury, while CTGF may serve to reconstruct extracellular matrix required for optimal re-epithelization. Taken together, the ability of γδ-T cells to produce CTGF, demonstrated herein, underscores the distinct role of this lymphocyte subpopulation in connective tissue reactions following injury.

Acknowledgments

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