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Orbital fibroblasts from patients with thyroid-associated ophthalmopathy over-express CD40: CD154 hyper-induces IL-6, IL-8 and MCP-1

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Abstract

Purpose—Fibroblast diversity represents an emerging concept critical to our understanding of tissue inflammation, repair and remodeling. Orbital fibroblasts heterogeneously display Thy-1 and exhibit unique phenotypic attributes that may explain the susceptibility of the human orbit to thyroid-associated ophthalmopathy (TAO). In the current study we investigate the role of CD40 ligation upon macrophage chemoattractant protein -1 (MCP-1), IL-6 and IL-8 expression in fibroblasts from patients with TAO.

Methods—Human orbital fibroblasts were cultured from tissues obtained with informed consent from patients with TAO and from patients undergoing surgery for other, non-inflammatory conditions. The fibroblasts were then examined by flow cytometry, microscopy, and cytokine assays.

Results—We report that orbital fibroblasts from patients with TAO express elevated levels of CD40. Surface CD40 can be further up-regulated by interferon- γ (IFN- γ) in both TAO and control fibroblasts. This up-regulation is mediated through Jak2 and can be blocked by dexamethasone and AG490, a powerful and specific inhibitor of the tyrosine kinase. Treatment with CD154, the ligand for CD40, up-regulates the expression of IL-6, IL-8 and MCP-1 in TAO fibroblasts, but fails to do so in control cultures. Thy-1⁺ fibroblasts displayed higher CD40 levels than do their Thy-1[−] counterparts and are largely responsible for this cytokine production. IL-1 β also induces MCP-1, IL-6 and IL-8 more vigorously in TAO-derived fibroblasts.

Conclusion—Characterization of orbital fibroblasts and their differential expression of cytokines and receptors should prove invaluable in understanding the site-specific nature of TAO and the development of specific therapies.

Introduction

Divergent phenotypic attributes of fibroblasts may help explain tissue-specific functions and anatomic site-selective vulnerability to disease. Fibroblast diversity represents an emerging concept potentially critical to our understanding of tissue inflammation, repair, and remodeling. This is true of systemic disease directed at the orbit, such as that occurring in Graves' disease (GD) and its orbital component, thyroid-associated ophthalmopathy (TAO). In TAO, lymphocytes, monocytes and mast cells infiltrate orbital tissues which become inflamed and extensively remodeled¹. Why mononuclear cells are trafficked to the orbit remains uncertain, but targeting these tissues may result from their unique immunological properties. In addition, physical peculiarities such as the bony orbit and its unique pattern of blood flow and lymphatic drainage might influence disease distribution^{2, 3}. Besides inflammation, the pathology of TAO involves accumulation of the non-sulfated glycosaminoglycan, hyaluronan⁴, fibrosis and increased fat volume⁵. The active phase of TAO is most frequently self-limited and can culminate in diminished eye motility⁶, orbital congestion and compressive neuropathy⁵. The potentially complex interplay between mononuclear cells and orbital fibroblasts may underlie the unusual tissue reactivity and remodeling occurring in TAO.

Orbital fibroblasts represent a heterogeneous population based on the cell-surface display of Thy-1, a membrane associated glycoprotein⁷. When sorted, into subsets, Thy-1⁺ fibroblasts express divergent cytokine profiles compared to their Thy-1⁻ counterparts following activation by proinflammatory molecules such as IL-1 β and CD154, the cognate ligand of CD40^{8, 9}. These subsets also possess distinct potential for terminal cell differentiation⁷. Thus, each fibroblast subset may serve distinct roles in health and disease.

CD40 and its cognate ligand CD154 represent an important activational pathway initially implicated in T cell/B cell interactions but more recently found important in communication between many cell-types, including endothelial cells, smooth muscle, fibroblasts, bone-marrow-derived and follicular dendritic cells^{10, 11}. CD40 is a member of the TNF- α receptor super-family which utilizes phosphorylation of TRAFs and NF κ B for cell signaling^{12, 13}. The interaction of CD40 on B cells with CD154-displaying activated T cells provides a costimulatory signal that induces T-dependent B cell proliferation and differentiation and leading to antibody production¹⁴. Professional antigen-presenting cells such as macrophages and dendritic cells require CD40 signaling for activation and utilize CD40 as a co-chaperone-like receptor, mediating the uptake of exogenous hsp70-peptide complexes¹⁰. Moreover, CD154 can act as a soluble cytokine¹⁵. Aberrant CD40-CD154 interactions appear to corrupt humoral immunity in autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis¹⁵.

In the current study, we assess several potentially important inflammatory attributes of orbital fibroblasts and their utilization of the CD40/CD154 molecular bridge. Previously, we have found that many of the actions of CD154 in orbital fibroblasts are mediated through the intermediate induction of IL-1 β , a cytokine that induces several cytokines and hyaluronan expression by orbital fibroblasts^{16, 17}. These include the induction of prostaglandin endoperoxide H synthase-2 (PGHS-2) gene expression¹⁸. In the current study we investigate the impact of CD154 on macrophage chemoattractant protein -1 (MCP-1), IL-6, and IL-8 expression in fibroblasts from patients with TAO. These findings help define more clearly the potential roles of fibroblast diversity in tissue reactivity and remodeling found in TAO.

Methods

Materials

Anti-Thy-1 and anti-CD40 Abs were purchased from BD Biosciences/Pharmingen (San Diego, CA). Rh IFN- γ and IL-1 β were obtained from BioSource (Camarillo, CA). AG490 was supplied by Calbiochem and dexamethasone was supplied by Sigma-Aldrich (St. Louis, MO). Culture medium and fetal bovine serum (FBS) were supplied by Gibco-Invitrogen (Carlsbad, CA). Recombinant CD154 expressing membranes and their controls were prepared as described previously¹⁹.

Cell Culture

Human orbital fibroblasts were cultivated as reported previously²⁰. Tissue explants were obtained from individuals undergoing orbital decompression for severe TAO or surgery for some other, non-inflammatory condition. These activities were undertaken after informed consent was obtained from the donors following procedures approved by the Institutional Review Boards of the Harbor-UCLA Medical Center/Los Angeles Biomedical Institute, Center for Health Sciences at UCLA, and according to the tenets of the Declaration of Helsinki. A total of 10 different fibroblast strains from patients with stable TAO and 4 control fibroblast strains were examined. Donors were euthyroid at the time of donation. Tissue explants attached to plastic culture dishes and were covered with medium containing glutamine and fetal bovine serum (FBS, 10%). They were incubated in a 37°C humidified incubator with a 5% CO₂ environment. Resulting fibroblast monolayers were serially passaged with gentle trypsin/EDTA treatment and were utilized for studies between the second and twelfth passage from culture initiation. Cultures were free from cells expressing factor VIII, α -smooth muscle-specific actin and cytokeratin²¹.

Preparation of Thy-1⁺ and Thy-1⁻ subsets

Separation of fibroblasts on the basis of Thy-1 display was conducted as reported previously²⁰. Briefly, nearly pure Thy-1⁺ and Thy-1⁻ subsets were generated following 3–4 rounds of magnetic bead selection^{22, 23}. Subsets exhibited a stable Thy-1 phenotype in culture, as monitored by flow cytometry and were either >99% Thy-1⁺ or >97% Thy-1⁻.

Methods for Immunostaining

Histologic sections from formalin fixed paraffin embedded tissue blocks were subjected to heat-induced epitope retrieval using a steamer at 95°C for 25 minutes in a 0.01M Citrate buffer, pH 6.0 for CD40, CD45, and smooth muscle actin. For factor VIII staining, slides were pretreated with proteinase K for 15 minutes at 37°C (Dako Corporation, Carpinteria, CA). The sections were incubated with mouse monoclonal antibody against CD40 (Novocastra, Bannockburn IL), or CD45 (Dako), respectively. Staining for smooth muscle actin was performed with rabbit anti α smooth muscle actin from Abcam Inc., Cambridge, MA, and factor VIII staining was performed with rabbit anti Von Willebrand Factor (factor VIII) (Dako). Following incubation with primary monoclonal antibody or polyclonal antiserum, localization of antigen was performed using the DakoCytomation EnVision+ System-HRP labeled. Following the diaminobenzidine reaction, the slides were counterstained in hematoxylin. Positive controls included tissues with known specificity for the antibodies (colon, tonsil) as well as vessels within the tissue which served as positive controls for factor VIII, smooth muscle actin, and CD45. Negative controls consisted of substitution of the primary antibody with isotype specific non-cross reacting monoclonal antibody (for the monoclonal antibody reagents) and species specific non-immune serum for the polyclonal antisera.

Flow cytometry

These techniques have been published⁷. Briefly, 1×10^6 cells were placed in 12×75 mm polypropylene tubes and fluorochrome-conjugated monoclonal antibodies ($1 \mu\text{g}/10^6$ cells) were incubated in the dark for 20 min at room temperature. Cells were washed twice with staining buffer (SB; phosphate buffered saline and 4% fetal calf serum), re-suspended, and maintained at 4°C until cytometric analysis (within 24 h) using a FACS Calibur flow cytometer (BD Biosciences). Mean fluorescent intensity (M.F.I.) was calculated as a ratio of mean fluorescence sample / isotype fluorescence.

Cytokine Assays

Fibroblasts were cultured to confluence, treated for 48 hours with or without $\text{RhIFN-}\gamma$ (100 U/ml) in MEM with 0.1% FBS, followed by treatment with $\text{IL-1}\beta$ (10 ng/ml), CD154 membranes, or control membranes for 48 hours. Previous work in our laboratory and that of others demonstrated these conditions provide maximal fibroblast response to these molecules^{9, 24, 25}. Culture medium was analyzed for IL-6 and IL-8 content using bead-based Luminex assays (Millipore). MCP-1 content was analyzed by standard sandwich ELISA (R & D Systems).

Statistics

Values are reported as the mean $\pm$ standard error. Statistical analysis was performed using a 2-tail Student's *t* test with a confidence level $>95\%$.

Results

TAO Orbital fibroblasts express higher levels of CD40 than those from control donors

Orbital fibroblasts from patients with TAO produce PGE_2 and hyaluronan in response to CD40 ligation in culture¹⁷. Thus we examined whether CD40 staining differed in tissues from patients with and without TAO. CD40^+ cells (arrows) were more abundant in disease-derived tissues (Figure 1B, C, D) than in controls (Figure 1A). CD40^+ staining was present in fibroblast-shaped cells and areas predominated by fat (Figure 1B, C, D). Infiltrating mononuclear cells and vascular structures also express CD40. However, sections stained with the leukocyte-specific marker, CD45 detected few infiltrating leukocytes (Figure 1E) and Factor VIII which stains endothelial cells was restricted to vessels (Figure 1F). CD40^+ cells were detected in areas devoid of Factor VIII and CD45 staining and were morphologically typical of fibroblasts.

We next examined CD40 display on isolated fibroblasts by flow cytometry (Figure 2, upper panel). The MFI of CD40 expression was 3.1 ± 0.3 ($n=7$) in disease-derived cells while those from controls was 1.1 ± 0.1 ($n=4$; $p < 0.001$) as compared to isotype control. Treatment with $\text{IFN-}\gamma$ (100 U/ml) for 72 h resulted in a dramatic up-regulation of CD40 in fibroblasts from both sources (MFI multiple 8.0 TAO; 6.4 control). Thus, CD40^+ fibroblasts are abundant in the orbital connective tissue from patients with TAO, and the receptor density on the cells *in vitro* is considerably greater than that found on controls. This divergence in receptor density suggests that TAO fibroblasts may interact with CD154-bearing T cells in a disease-specific manner. Since $\text{IFN-}\gamma$ has been detected in active TAO, we assessed the mechanism through which it up-regulates CD40^{26, 27}. We focused on Jak2 since that kinase plays a central role in signaling found in TAO fibroblasts¹⁸. In the presence of AG490 ($75 \mu\text{M}$, a specific inhibitor of Jak2), $\text{IFN-}\gamma$ -dependent CD40 expression by both TAO and control fibroblasts was markedly reduced to levels observed in the absence of cytokine (Figure 2, lower panel). Treatment with dexamethasone (10 nM) also inhibited the $\text{IFN-}\gamma$ -induction of CD40. Thus, interfering with Jak2 signaling appears to markedly attenuate CD40 expression induced by $\text{IFN-}\gamma$ in both TAO and control fibroblasts. On the other hand, the elevated basal CD40 levels found in TAO fibroblasts were unaffected by AG490 and dexamethasone, suggesting that Jak2

does not support the elevated constitutive receptor display found in TAO (Figure 2, lower panel).

We next assessed whether high-level CD40 expression was global and distributed widely among fibroblast subsets segregated on the basis Thy-1 display or confined to a particular subset. CD40 levels in mixed populations were compared with those of sorted fibroblast subsets and found to be highly expressed by parental TAO fibroblasts (MFI 3.2 ± 0.3 fold greater than isotype) compared to their control counterparts (MFI 1.1 ± 0.2 fold greater than isotype) (Figure 3). CD40 was minimally expressed in the Thy-1⁺ and Thy-1⁻ control populations after sorting and culture (Figure 3, upper panel). However, its expression in TAO fibroblasts was found predominantly in the Thy-1⁺ subset (Thy-1⁺ MFI 3.4 ± 0.3 fold compared to Thy-1⁻ MFI 1.4 ± 0.3 fold; $p < 0.05$, $n = 4$).

CD154 induces IL-6, IL-8, and MCP-1 in Thy-1⁺ TAO orbital fibroblasts

We determined the functional consequences of CD40 display by incubating control and TAO cultures with CD154-expressing insect membranes or control membranes and assessed IL-6 and IL-8 production resulting from each treatment. The parental strain, comprising 55% Thy-1⁺ and 45% Thy-1⁻ fibroblasts, and its derivative subsets were stimulated with CD154 for 48h. Figure 4 demonstrates IL-6 and IL-8 over-production in parental TAO strains when compared to control cultures following CD154 treatment (representative of 4 replicates; TAO vs control, $*p < 0.01$). The TAO Thy-1⁺ subset also exhibited substantial IL-6 and IL-8 production while that in Thy-1⁻ fibroblasts was considerably less ($*p < 0.01$). Thus, TAO-derived Thy-1⁺ cells appear more responsive to CD154 than do their Thy-1⁻ counterparts with regard to both IL-6 and IL-8 expression. In contrast, control fibroblasts produced low IL-6 and IL-8 levels under these same conditions. Thus, the higher levels of CD40 found in Thy-1⁺ TAO fibroblasts may be critical to the generation of IL-6 and IL-8 in response to CD154. Given the role of immune cell infiltration in this disease, we also examined the expression of MCP-1. Control fibroblasts expressed modest MCP-1 under basal culture conditions and the levels were not induced by CD154 (Figure 5). In contrast, the TAO parental strain and both sorted subsets expressed increased levels of MCP-1 in response to CD154 ($n = 4$, $*p < 0.01$). Thus, in contrast to IL-6 and IL-8 expression, the magnitude of MCP-1 induction is not proportional to CD40 expression levels by Thy-1⁺ and Thy-1⁻ TAO subsets.

IL-1 β also induces IL-6 and IL-8 production to a considerably higher level in TAO fibroblasts compared to controls

Given the divergent phenotypic and functional properties of TAO orbital fibroblasts with regard to the CD40/CD154 bridge, we explored whether responses of these cells might be generalized to other cytokines important in TAO. IL-1 β appears to have a central role in TAO by inducing cytokine and extracellular matrix production in a site-selective manner^{16,18}. Moreover, IL-1 α is induced by CD154 in TAO fibroblasts and serves as a critical intermediate for the induction of PGHS-2²⁴. As shown in Figure 6, several strains of TAO fibroblasts express considerably more IL-8 in response to IL-1 β than do control cultures. However, TAO and control fibroblasts produced similar levels of IL-6. Thus, the exaggerated production of pro-inflammatory cytokine production in orbital fibroblasts in response to other cytokines appears to be cell-type and agent-specific.

Discussion

CD40 and its ligand, CD154, represent an important cell activation pathway²⁸. We have previously reported that CD40 activation in orbital fibroblasts results in the induction of PGHS-2 and the production of PGE₂ and hyaluronan^{29, 30}. Here we demonstrate that unprovoked orbital fibroblasts from patients with TAO express substantially higher levels of

CD40 than control fibroblasts. When treated with IFN- γ , CD40 levels are substantially increased in fibroblasts from both sources, an action mediated by Jak2.

We demonstrate that TAO orbital fibroblasts produce significantly more IL-6, IL-8 and MCP-1 through CD40 ligation when compared to orbital fibroblasts from control donors. Elevated serum IL-8 levels were found previously in hyperthyroid patients with GD and these may reflect production in sites other than the thyroid³¹. In this manuscript, we demonstrate that CD40/CD154 and IL-1 β may enhance IL-8 production in the orbit. High serum IL-6 levels are also associated with GD and we have found that IL-1 β increases production of this cytokine uniquely in orbital fibroblasts. {Celik, 1995 #4841}{Hiromatsu, 2000 #2120}{Bossowski, 2001 #3517} Local levels in the orbit may promote lymphocyte activation or recruitment³². IL-6 induces a number of B cell genes, and its actions are generally associated with anti-apoptotic effects and Ig production. Moreover, it drives the synthesis of Igs and is necessary for the normal development of plasma cells³³. Thus, it remains possible that B cells in the orbit might overproduce Igs, such as those associated with GD, as a consequence of the high IL-6 levels generated by fibroblasts. With regard to T cells, IL-6 promotes IL-4 synthesis and Th2 development through transcriptional activation of NFAT^{34, 35}. CD40 interactions with T cells may provide a Th2 type microenvironment. It may also directly promote T cell migration, effects that are mediated through MAPK, PI₃K, and the Jak/STAT pathways³⁶. Thus, our findings here that activation of the CD40/CD154 pathway also induces IL-6 and IL-8 suggest that the activation of multiple pathways might underlie the characteristic pattern of inflammatory responses seen in TAO.

MCP-1 is a powerful chemoattractant that targets mononuclear infiltration and promotes inflammation^{31, 37}. Moreover, it has been implicated previously in human autoimmune disease³⁸. Macrophage infiltration is a characteristic feature of orbital fat reactivity in active TAO and localizes around blood vessels and between mature adipocytes³¹. MCP-1 mRNA is more abundant in TAO orbital fat although protein levels *in vivo* are difficult to quantify³¹. We were unable to detect the MCP-1 protein in tissue preparations, but this may be reflective of the relatively late stage of disease progression in which these samples were obtained since macrophage infiltration was also minimal (unpublished observation). Previous reports have demonstrated that IL-1 β can induce MCP-1 in a variety of cell types, effects that can be blocked by dexamethasone^{39, 40}. Here we demonstrate that MCP-1 is also induced in response CD40 ligation specifically in TAO orbital fibroblasts. Thus, the cytokine may play an important role in macrophage recruitment in the early disease.

Insinuating Jak2 into the induction of CD40 by IFN- γ represents a new insight. We have very recently demonstrated the importance of Jak2 in the modulation of PGHS-2 induction by IL-1 β in orbital fibroblasts from TAO patients¹⁸. There is coordinate induction of PGHS-2 and PGE₂ in these cells when treated with proinflammatory cytokines such as CD154 and IL-1 β which may contribute to site-specific inflammation. Our finding demonstrating the role of Jak2 in INF- γ -dependent CD40 expression represents a potentially important component of the inflammatory phenotype of these cells.

Features associated with GD and TAO suggest that peculiarities in fibroblast phenotype might underlie susceptibility to tissue remodeling. This possibility has prompted a number of studies examining the cellular characteristics of orbital fibroblasts^{24, 41}. The relatively high-density surface display of CD40 by TAO orbital fibroblasts suggest that they might interact efficiently with recruited immuno-competent cells such as T lymphocytes. We postulate that their enhanced ability to cross-talk with these cells should promote orbital tissue activation. We have previously demonstrated several unique functional attributes of orbital fibroblasts derived from patients with TAO. These include the exaggerated up-regulation of hyaluronan, plasminogen activator inhibitor 1^{32, 42}, tissue inhibitor of metalloproteinase¹⁶, PGHS-2¹⁸, UDP glucose

dehydrogenase⁴ and 15-lipoxygenase-1⁴³. These findings, all made *in vitro* using cells derived from primary human tissue, must be interpreted with caution since no animal models of TAO currently exist. Despite these limitations, our current findings identify features that may prove common to the regulation of these and many other genes. Thus, they hold the potential in aggregate for both explaining the tissue specificity of TAO and identifying a cellular feature that might be exploited for therapeutic development, such as the signaling associated with the Jak2 pathway..

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Figure 1. Immunostaining of CD40 in control orbital tissue and TAO. Fibroblast-like CD40⁺ cells (arrows) are more numerous in the stromal and fat predominant areas of thin sections from a patient with TAO (B,C,D) compared to control tissue (A). (E) Minimal expression of CD45 in sections from disease-derived tissue. (F) Factor VIII staining is restricted to vascular endothelium

Figure 2.

CD40 displayed by orbital fibroblasts and its up-regulation with IFN- γ . (upper panel) TAO and control fibroblasts were cultured as described in “Methods”. They were stimulated with or without IFN- γ (100 U/ml) for 48 hours and stained with anti-CD40 or its isotype (open histogram dark grey) Abs. (Lower panel) Fibroblasts were incubated with IFN- γ with or without AG490 (75 μ M) or dexamethasone (10 nM) for 72 hours. CD40 expression was assessed as the multiple of constitutive expression.

Figure 3.

Thy-1⁺ fibroblasts from patients with TAO express CD40. Parental fibroblast strains were sorted based upon Thy-1 display and cultured. Subsets were then stained for CD40 expression. Un-stimulated fibroblasts were stained with an isotype-control Ab (open histogram dark grey) or anti-CD40 (open histogram light grey) while others were treated with IFN-γ (100 U/ml) for 48 hours and stained with anti-CD40 Abs (black histogram).

Figure 4.

CD154 induces IL-6 and IL-8 in Thy-1⁺ TAO orbital fibroblasts. Fibroblasts from donors with TAO and those without disease were isolated, cultured, and some treated with IFN- γ for 48h followed by incubation with CD154 membranes for 48h. Concentrations of IL-6 (panel A) and IL-8 (panel B) were determined in parental, Thy1⁺ and Thy1⁻ subsets. (* p<0.01, representative of 4 experiments).

Figure 5. CD154 induces MCP-1 in Thy-1⁺ and Thy-1⁻ orbital fibroblasts from donors with TAO. Cultures were isolated and treated for 48 hours with IFN- γ and then with CD154 or control membranes for an additional 48h. MCP-1 concentrations were determined in parental strains and those sorted into Thy-1⁺ and Thy-1⁻ subsets. (* p < 0.01, representative of 4 experiments).

Figure 6.

IL-1 β induces IL-6 and IL-8 in orbital fibroblasts. Fibroblasts from donors with TAO and controls were stimulated with IL-1 β (10 ng/ml) for 48h. Concentrations of IL-6 (panel A) and IL-8 (panel B) were determined. (* p < 0.01, representative of 4 experiments).