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Synergistic Effect of TNF-α and IL-4 on the Expression of Thymus- and Activation-Regulated Chemokine in Human Corneal Fibroblasts

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Chemokine production by resident fibroblasts contributes to the recruitment of migratory leukocytes to sites of tissue injury. The effects of cytokines on the expression of thymus- and activation-regulated chemokine (TARC), a potent chemoattractant for Th2 cells, were examined in cultured human corneal fibroblasts. The culture supernatants of cells incubated for 24 h in the absence or presence of either TNF-α (10 ng/ml) or IL-4 (10 ng/ml) alone did not contain TARC detectable by ELISA. However, exposure of cells to both cytokines resulted in marked release of TARC into the culture medium in a time- and dose-dependent manner. Similarly, quantitative RT-PCR analysis revealed that the two cytokines induced a synergistic increase in the amount of TARC mRNA in the cultured fibroblasts. This synergistic effect of TNF-α and IL-4 on TARC production by fibroblasts may contribute to the Th2 cell infiltration and consequent tissue damage associated with allergic inflammation.

Key Words: **human; fibroblasts; allergy; cytokines; chemokines.**

Thymus- and activation-regulated chemokine (TARC)[2] is a member of the C-C chemokine family and is a potent chemoattractant for Th2 cells, which express the CCR4 receptor for TARC on their surface (1, 2). Cytokines secreted by activated Th2 cells, such as IL-4, IL-5, and IL-10, induce the release of IgE from B cells and activate inflammatory effector cells, resulting in tissue destruction. The local production of TARC therefore plays an important role in the induction and maintenance of allergic reactions (3) as a result of the promotion by this chemokine of Th2 cell infiltration (4, 5). Such local infiltration of Th2 cells is pronounced in individuals with atopic dermatitis or keratoconjunctivitis (6, 7).

Fibroblasts are major structural components of tissue and are also thought to be responsible for the local recruitment of inflammatory cells (8) as a result of their ability to produce a variety of chemokines (9–11). Allergic reactions are triggered by the activation and degranulation of mast cells, which result in the release of cytokines that include the Th2 cytokine IL-4 and the proinflammatory cytokine TNF-α. We and others recently demonstrated synergistic effects of IL-4 and TNF-α on eotaxin expression in corneal and skin fibroblasts (9, 12). However, the effects of these cytokines on the possible production and release of TARC by fibroblasts have remained unknown.

To shed light on the mechanism by which local infiltration of Th2 cells is regulated, we have therefore now investigated whether cultured human corneal fibroblasts produce TARC and the possible effects of IL-4 and TNF-α on such production.

MATERIALS AND METHODS

Materials. Eagle MEM was obtained from the Research Foundation for Microbial Diseases of Osaka University (Osaka, Japan); OPTI-MEM, FBS, and trypsin (0.05%)–EDTA (0.53%) were from Gibco-BRL (Grand Island, NY); and tissue culture dishes (Falcon) were from Becton–Dickinson (Franklin Lakes, NJ). Human recombinant TNF-α and IL-4 were obtained from Genzyme (Cambridge, MA). Paired antibodies to TARC and human TARC protein standard for ELISA were obtained from R&D Systems (Minneapolis, MN). The RNeasy Mini Kit was from Qiagen (Hilden, Germany), the TaKaRa One Step RNA PCR Kit (AMV) was from Takara Shuzo (Shiga, Japan), LightCycler-DNA Master SYBR Green I was from Roche Molecular Biochemicals (Mannheim, Germany), ethidium bromide and DNA molecular size standards (Marker 11) were from Nippongene (Toyama, Japan), and agarose (NuSieve 3:1) was from FMC Bioproducts (Rockland, ME). All reagents used for cell culture were endotoxin minimized.

Isolation, culture, and stimulation of human corneal fibroblasts. Four human corneas were obtained from Mid-America Transplant Service (St. Louis, MO) and NorthWest Lions Eye Bank (Seattle,

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[2] Nonstandard abbreviation: TARC, thymus- and activation-regulated chemokine.

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WA). The donors were Caucasian men and women ranging in age from 4 to 65 years. After the center of each donor cornea was punched out for corneal transplantation surgery, the remaining rim of the cornea was used for the present experiments. The human tissue was used in strict accordance with the basic principles of the Declaration of Helsinki. Each cornea was digested separately with collagenase to provide a suspension of corneal fibroblasts (13). The cells from each cornea were cultured separately in MEM supplemented with 10% FBS, and they were used for the present studies after four to six passages. The purity of the cultured cells was judged on the basis of both the morphology of corneal fibroblasts and their reactivities with antibodies to vimentin and cytokeratin in immunofluorescence analysis (9, 11). All the cells were positive for vimentin and negative for cytokeratin, suggesting the absence of contamination of the cultures by epithelial cells. No change in cell morphology or immunoreactivity was apparent after culture for four to six passages.

Cells (3×10^5) were seeded in 60-mm dishes and grown until they became confluent. They were stimulated with cytokines as described previously (9, 11). In brief, the culture medium of the confluent cells was replaced with OPTI-MEM, and the cells were incubated for 3 days. The medium was then changed to OPTI-MEM supplemented with TNF-α or IL-4 (or both). After incubation for the indicated periods of time, the medium was collected from each culture dish and centrifuged at 120*g* for 5 min. The resulting supernatant was stored at −80°C for subsequent assay of TARC. The fibroblasts remaining in the dish were exposed to trypsin-EDTA, and their number was determined with a hemocytometer.

Determination of TARC protein concentration by ELISA. The concentration of TARC in culture supernatants was determined in triplicate with a solid-phase ELISA. The limit of detection was 3.9 pg/ml, and data were expressed as picograms of TARC per 10^6 cells.

Quantitative RT-PCR analysis of TARC mRNA. Corneal fibroblasts were cultured and stimulated as described above, after which the cells were washed with PBS and total RNA was extracted with a kit. Extracted RNA was subjected to reverse transcription with a kit and the abundance of TARC mRNA was quantified by real-time PCR with a LightCycler instrument (Roche Molecular Biochemical). Transcripts of the constitutively expressed gene for GAPDH served to normalize the amount of TARC mRNA in each sample. The sequences of the PCR primers were as follows: TARC sense, 5′-CTCCTCCTGGGGGCTTCTCT-3′; TARC antisense, 5′-GTTGGGGTCCGAACAGATGG-3′; GAPDH sense, 5′-GCCAAAAGGGTCATCATCTC-3′; and GAPDH antisense, 5′-ACCACCTGGTGCTCAGTGTA-3′. These primers yielded PCR products of the expected sizes of 198 bp for TARC mRNA and 500 bp for GAPDH mRNA (14, 15).

The PCR protocol comprised an initial denaturation step at 95°C for 30 s followed by 40 cycles of amplification. For the amplification of TARC cDNA, the cycles consisted of denaturation at 95°C for 15 s, annealing at 59°C for 10 s, and elongation at 72°C for 10 s. For the amplification of GAPDH cDNA, the cycles included denaturation at 95°C for 15 s, annealing at 55°C for 10 s, and elongation at 72°C for 20 s. Real-time PCR data were analyzed with LightCycler software 3.01 (Roche Molecular Biochemicals). To verify the specificity of the amplification, we also subjected PCR products to electrophoresis on a 3% agarose gel, which was then stained with ethidium bromide (1 μg/ml) and examined with a Nighthawk system (pdi, Huntington Station, NY). The latter comprised a charge-coupled device camera, an ultraviolet transilluminator, and an analysis program (Quantity One).

Statistical analysis. Data are expressed as means ± SEM. Differences were analyzed by Student's *t* test or by ANOVA and Fisher's PLSD test. A *P* value of <0.01 was considered statistically significant.

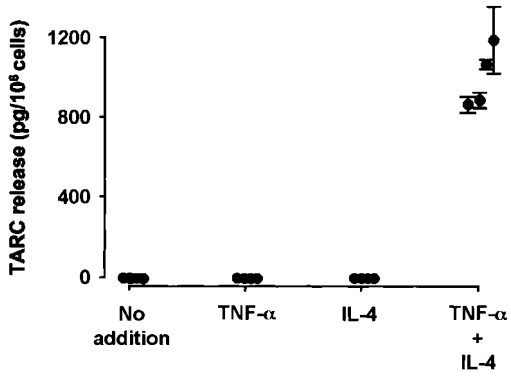

FIG. 1. Effects of TNF-α and IL-4 on TARC production by human corneal fibroblasts derived from different donors. Corneal fibroblasts prepared from four different donors were incubated separately for 24 h in the absence or presence of TNF-α (10 ng/ml), IL-4 (10 ng/ml), or both cytokines, after which the concentration of TARC in the culture supernatant was measured by ELISA. Data are means ± SEM of values from four separate experiments, each performed with four replicates, for each donor.

RESULTS

Synergistic Effect of TNF-α and IL-4 on TARC Release by Human Corneal Fibroblasts

We first examined the effects of TNF-α and IL-4 on the production of TARC by human corneal fibroblasts isolated from four different donors. The cells were cultured for 24 h with each cytokine at a concentration of 10 ng/ml either separately or in combination. TARC was not detectable in the culture medium of cells incubated in the absence of cytokine or in the presence of TNF-α or IL-4 alone (Fig. 1). However, incubation of cells with the combination of TNF-α and IL-4 induced a marked increase in the release of TARC by all four cell preparations. Given that the responses of cells from the four different donors were virtually identical, we performed subsequent experiments with corneal fibroblasts from one donor.

We next investigated the time course of TARC release by corneal fibroblasts incubated for up to 24 h with TNF-α (10 ng/ml), IL-4 (10 ng/ml), or the combination thereof. Whereas incubation of cells with TNF-α or IL-4 alone did not induce detectable release of TARC during the incubation period (data not shown), exposure of the corneal fibroblasts to the combination of TNF-α and IL-4 resulted in a time-dependent increase in TARC release that was statistically significant at 12 and 24 h (Fig. 2).

The dose dependence of the effect of the combination of TNF-α and IL-4 on the production of TARC by corneal fibroblasts was then examined. Cells were cultured for 24 h with various concentrations of TNF-α in the absence or presence of IL-4 (10 ng/ml) (Fig. 3) or with various concentrations of IL-4 in the absence or presence of TNF-α (10 ng/ml) (Fig. 4). In the absence of

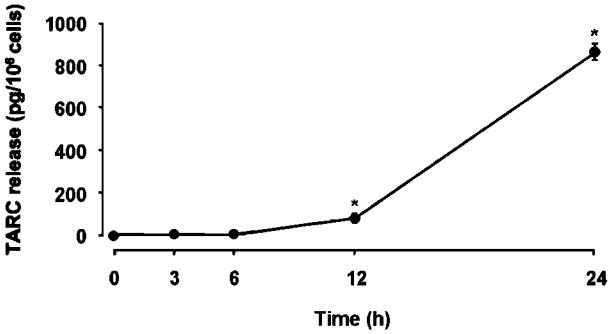

FIG. 2. Time course of TARC release from human corneal fibroblasts stimulated with the combination of TNF-α and IL-4. Cells were cultured for the indicated times in the presence of both TNF-α (10 ng/ml) and IL-4 (10 ng/ml), after which the culture supernatant was assayed for TARC by ELISA. Data are means ± SEM (n = 4) from a representative experiment that was repeated three times with similar results. *$P < 0.01$ (Student's t test) vs the zero time point.

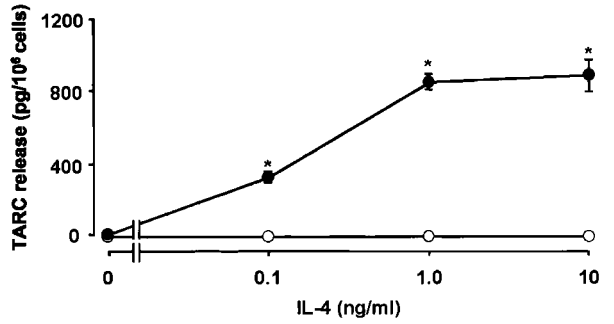

FIG. 4. Dose dependence of the effect of IL-4 in the absence or presence of TNF-α on TARC release by human corneal fibroblasts. Cells were cultured for 24 h with the indicated concentrations of IL-4 in the absence (open circles) or presence (closed circles) of TNF-α (10 ng/ml). Culture supernatants were then assayed for TARC by ELISA. Data are means ± SEM (n = 4) from a representative experiment that was repeated four times with similar results. *$P <$ 0.01 (Fisher's PLSD test) vs the corresponding value for cells incubated in the absence of TNF-α.

IL-4, corneal fibroblasts did not release detectable amounts of TARC into the culture medium at any concentration of TNF-α; however, in the presence of IL-4, TNF-α induced a dose-dependent increase in TARC release that was statistically significant at concentrations of 0.1, 1, and 10 ng/ml (Fig. 3). Similarly, in the absence of TNF-α, IL-4 did not induce detectable release of TARC; however, in the presence of TNF-α, IL-4 induced a dose-dependent increase in TARC release that was statistically significant at concentrations of 0.1, 1, and 10 ng/ml (Fig. 4).

Synergistic Effect of TNF-α and IL-4 on the Abundance of TARC mRNA in Corneal Fibroblasts

Finally, we investigated the effects of TNF-α and IL-4 on the abundance of TARC mRNA in human corneal fibroblasts. Cells were cultured for 24 h in the absence or presence of cytokines, each at a concentration of 10 ng/ml, after which the amount of TARC mRNA in cell lysates was assayed by quantitative RT-PCR. Exposure of cells to TNF-α or IL-4 alone had no significant effect on the amount of TARC mRNA. In contrast, stimulation with both TNF-α and IL-4 induced an ~500-fold increase in the abundance of TARC mRNA compared with that apparent in unstimulated cells (Fig. 5).

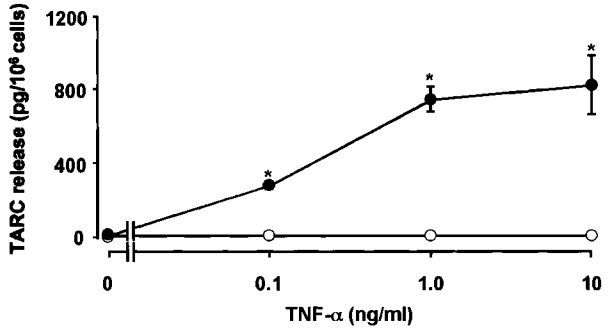

FIG. 3. Dose dependence of the effect of TNF-α in the absence or presence of IL-4 on TARC release by human corneal fibroblasts. Cells were cultured for 24 h with the indicated concentrations of TNF-α in the absence (open circles) or presence (closed circles) of IL-4 (10 ng/ml). Culture supernatants were then assayed for TARC by ELISA. Data are means ± SEM (n = 4) from a representative experiment that was repeated three times with similar results. *$P <$ 0.01 (Fisher's PLSD test) vs the corresponding value for cells incubated in the absence of IL-4.

DISCUSSION

We have shown that the combination of TNF-α and IL-4 induces a synergistic increase in the expression of

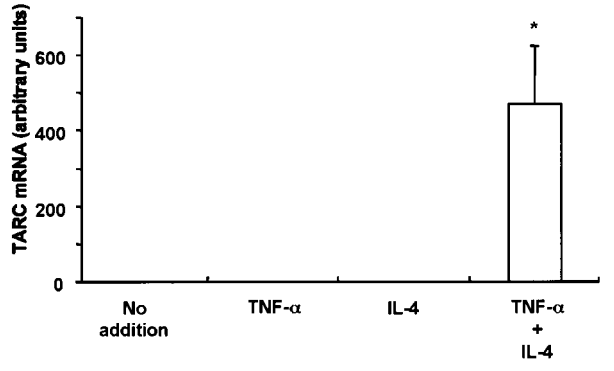

FIG. 5. Effects of cytokines on the abundance of TARC mRNA in human corneal fibroblasts. Cells were cultured for 24 h in the absence or presence of TNF-α (10 ng/ml), IL-4 (10 ng/ml), or the combination thereof, after which cell lysates were assayed for TARC mRNA by quantitative RT-PCR. The amount of TARC mRNA was normalized by that of GAPDH mRNA and is presented in arbitrary units. Data are means ± SEM (n = 3) from a representative experiment that was repeated four times with similar results. *$P < 0.01$ (Fisher's PLSD test) vs the corresponding value for cells incubated in the absence of cytokines.

TARC at both the protein and mRNA levels in cultured human corneal fibroblasts. Exposure of the cells to either cytokine alone had no effect on TARC expression. Given that TARC is a potent chemoattractant for Th2 cells, our results suggest that cytokine-induced activation of fibroblasts may contribute to the pathogenesis of Th2 cell-mediated allergic reactions in ocular tissue by promoting the infiltration of Th2 cells. To determine whether TNF-α and IL-4 also induce TARC production in fibroblasts derived from other tissues, we also investigated the effects of these cytokines in cultured human skin fibroblasts. The combination of TNF-α and IL-4 indeed induced synergistic increases in TARC release and in the amount of TARC mRNA in skin fibroblasts (unpublished data).

The local concentrations of IL-4 and TNF-α are important determinants of Th2 cell infiltration. Thus, IL-4 is required for the local recruitment of Th2 cells in the lung (14), and the local administration of IL-4 induces Th2 cell infiltration *in vivo* (15). Local administration of TNF-α promotes the transendothelial migration of Th2 cells and potentiates mucosal inflammation in the airway epithelium (14). However, the mechanism by which IL-4 or TNF-α induces Th2 cell infiltration has remained unclear, given that these cytokines have not been shown to act as direct chemoattractants for Th2 cells *in vitro*. Our results now suggest that the effects of IL-4 and TNF-α on Th2 cell infiltration might be mediated by the induction of TARC production by fibroblasts.

The activation and degranulation of mast cells are the first events in an allergic reaction. Activation of these cells by antigen-mediated cross-linking of IgE expressed on the cell surface results in their expression of IL-4 and TNF-α (16, 17). Thus, it is possible that IL-4 and TNF-α released from activated mast cells induce the production of TARC by fibroblasts and thereby trigger the local infiltration of Th2 cells. IL-5 is another Th2-type cytokine released by activated mast cells (18). We therefore also examined the effect of IL-5 on TARC expression by human corneal fibroblasts; however, this cytokine, either alone or in combination with TNF-α, did not induce a detectable level of TARC release by these cells (unpublished data).

Synergistic effects of IL-4 and TNF-α on fibroblasts appear to be important in the pathogenesis of allergic reactions. The combination of these cytokines induces the expression by fibroblasts of eotaxin (9, 12), which is a potent and specific chemoattractant for eosinophils (19). In addition, these two cytokines induce the expression by fibroblasts of the adhesion molecule VCAM-1, which is a ligand for VLA-4 expressed on the surface of eosinophils and lymphocytes (19). These observations, together with the results of the present study, indicate that activation of fibroblasts by the combination of IL-4 and TNF-α is an important step in the initiation and maintenance of Th2-type immune responses.

The cornea is an avascular tissue that is composed of only three different cell types: epithelial cells, fibroblasts, and endothelial cells. Corneal fibroblasts are the main source of chemokines in this tissue, having been shown to express IL-8, RANTES, and eotaxin (9–11). We have now added TARC to this list. We have also shown that IL-4, IL-5, and TNF-α have no effect on TARC production or the abundance of TARC mRNA in SV40-transformed human corneal epithelial cells (unpublished data). Although the clinical importance of TARC in ocular allergic reactions has not been defined, both Th2 cell infiltration in biopsy specimens and increased concentrations of TNF-α (20) and IL-4 (21) in tear fluid of individuals with severe allergic conjunctivitis have been described. It is thus possible that TARC produced by corneal fibroblasts in response to stimulation with IL-4 and TNF-α present in tear fluid is responsible for Th2 cell recruitment in severe ocular allergic reactions.

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