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Rapid Communication

Neutrophil Chemotactic Factor (IL-8) Gene Expression by Cytokine-treated Retinal Pigment Epithelial Cells

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The neural-derived retinal pigment epithelium (RPE) underlies the sensory retina and is central to both retinal homeostasis and many common retinal diseases. Retinal pigment epithelium cells are actively phagocytic and share several features with macrophages that have recently been shown to produce a neutrophil chemotactic factor (NCF), also known as interleukin-8, after cytokine stimulation. Because RPE cell responses to cytokines are largely unknown, human RPE cell NCF production was monitored after interleukin-1-beta (IL-1β), tumor necrosis factor-alpha, or lipopolysaccharide stimulation. RPE NCF mRNA expression and RPE production of biologically active NCF was time and concentration dependent. Maximal NCF mRNA expression occurred at 20 ng/ml for IL-1β. Messenger RNA expression in RPE cells and biologically active NCF in RPE cell supernatants were found 1 hour after stimulation and were maintained for 24 hours. These findings demonstrate that cytokine-stimulated RPE cells may evoke or augment neutrophil-mediated inflammation by synthesizing NCF, a cytokine that may be important in ocular disease mechanisms. (Am J Pathol 1990, 136:745–750)

The neural-derived retinal pigment epithelium (RPE) forms a monolayer of cells interposed between the neurosensory retina and circulating blood within the choroid.[1] In this strategic location, the RPE forms a part of the blood–retina barrier,[1] performs functions essential to retinal integrity and function,[2] and plays important roles in vascular, inflammatory, degenerative, and dystrophic diseases of the retina and choroid.[3–6] Recent investigations have shown that RPE cells possess several macrophagelike features and functions, including the presence of Fc and C3 receptors, acetyl and native low-density lipoprotein receptors, and prominent acid lipase activity.[7] In this study, we report that cytokine-stimulated RPE cells synthesize and secrete a neutrophil chemotactic factor (NCF) with characteristics similar, if not identical, to NCF (interleukin-8; IL-8) derived from cytokine-stimulated monocytes.[8] Neutrophil chemotactic factor production by resident, neural-derived RPE cells in response to cytokines secreted by inflammatory cells, vascular endothelial cells, fibroblasts, and astrocytes may be important in the initiation and propagation of ocular diseases.

Materials and Methods

RPE Cell Culture

RPE cells were isolated from human eyes and cultured in Dulbecco's Modified Essential Medium supplemented with 15% fetal bovine serum.[9] Human RPE cell cultures consisted of cell monolayers exhibiting typical hexagonal cellular arrays that stained immunohistochemically for fi-

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bronectin, laminin, and type IV collagen.[10] Sixth passaged cells were assayed. Before stimulation, RPE cells were rinsed and placed in fresh, serum-free medium.

RPE Cell Stimulation with IL-1β, TNF-α, and LPS

Retinal pigment epithelium cells were left unstimulated or treated with human recombinant interleukin-1-beta (IL-1β), human recombinant tumor necrosis factor-alpha (TNF-α), or lipopolysaccharide (LPS; *Escherichia coli* 0111:B4, Sigma Chemical Co, St. Louis, MO). The specific activities of IL-1β (Upjohn Co., Kalamazoo, MI) and TNF-α (Cetus Corp., Emeryville, CA) were 30 U/ng and 20 U/ng, respectively. TNF-α was expressed and purified as described previously.[11] After these incubations, culture medias were collected, centrifuged to remove particulates, and stored at −70 C until neutrophil chemotactic activity was assayed.

RPE Cell NCF mRNA Analysis

Total cellular RNA was extracted by a modification of the method of Chirgwin et al[12] and Jonas et al.[13] A 30-nucleotide probe to human NCF was synthesized using cDNA complementary to nucleotides 262 to 291; its sequence was: 5′-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3′.[8] The probe was 5′-end labeled with [^{32}P]adenosine 5′-triphosphate (ICN Biomedicals, Inc., Costa Mesa, CA). Specific activity of the probe was 6.5 × 10^8 cpm/μg. Extracted RNA was separated by electrophoresis, transferred to nitrocellulose, hybridized with the ^{32}P-labeled probe, and washed in 6x standard saline citrate (SSC) and 0.5% sodium pyrophosphate at 57 C for 1 hour. Blots were autoradiographed and quantitated by laser densitometry. Equivalent amounts of total RNA/gel was assessed by monitoring 28 and 18 s rRNA.

NCF Bioactivity Assays

Assays for chemotactic bioactivity were performed in blind, multiwell Boyden chemotactic chambers (Neuroprobe Inc., Cabin John, MD).[14] Hanks Balanced Salt Solution alone (225 μl), or 50% dilutions of culture supernatants, or 10^{-7} mol/l (molar) formylmethionylleucylphenylalanine (fMLP, Sigma) were placed in bottom wells. Three-micrometer polycarbonate filters (polyvinylpyrrolidone-free, Nuclepore Corp., Pleasanton, CA) were placed in the assemblies, and neutrophils (333 μl; 2 × 10^6 cells/ml) were added to top chambers. After 60 minute incubations at 37 C in a humidified chamber, filters were removed, fixed with methanol, and stained with 2% toluidine blue. Neutrophils that had migrated to the bottoms of the filters were counted in 10 high-power fields. IL-1β– or TNF-α–induced bioactivity is expressed as a percentage of neutrophil chemotaxis caused by 10^{-7} mol/l fMLP.

Specificity of RPE-derived NCF activity was established by preincubating aliquots of the above supernatants with specific anti-human rNCF antibody[15] (1:100), which blocked the bioactivity of the RPE supernatants by 60%. Preincubations with preimmune serum did not block any chemotactic activity in parallel assays. Chemotactic activity could not be detected in unstimulated RPE cultures assayed after 24 hours.

Results

RPE NCF mRNA expression, measured by Northern blot analysis, was induced 1 hour after challenge with IL-1β or TNF-α, was time dependent, and was not detected in unstimulated RPE cells (Figure 1). In contrast, LPS failed to induce significant NCF mRNA in RPE cells until 24 hours. Peak NCF mRNA expression occurred at 24 hours for IL-1β and at 4 hours for TNF-α. IL-1β was a more potent stimulator of NCF mRNA than either TNF-α or LPS.

NCF bioactivity after IL-1β and TNF-α stimulation was detected in RPE cell supernatants 1 hour after challenge and was present for at least 24 hours after stimulation (Figure 2). Peak NCF biologic activities in the RPE cell conditioned media (CM) were found at 24 hours, presumably from accumulating NCF product corresponding to continued NCF mRNA induction. Neutrophil chemotactic factor activity in the supernatants was inhibited 60% by incubation with specific anti-NCF antibody,[15] but not by incubation with preimmune serum.

To distinguish chemotactic activity from chemokinesis, a checkerboard analysis was performed using 24-hour CM from RPE cells stimulated with 20 ng/ml of IL-1β, as shown in Table 1. Conditioned media in varying concentrations was added to the top and bottom wells of a multiwell chemotaxis chamber, with neutrophils placed in the top wells. Neutrophil migration occurred in a concentration-dependent manner. More neutrophils migrated to bottom wells containing a higher concentration of CM. When the CM concentration was equivalent in the top and bottom wells, minimal neutrophil migration occurred.

Steady-state NCF gene expression after IL-1β stimulation was also concentration dependent (Figure 3). Maximal NCF mRNA expression occurred at an IL-1β concentration of 20 ng/ml and detectable expression was observed at 20 pg/ml. Fifty percent of the maximal response occurred between 0.2 and 2 ng/ml of IL-1β. Neutrophil chemotactic factor bioactivity in RPE cell supernatants 4

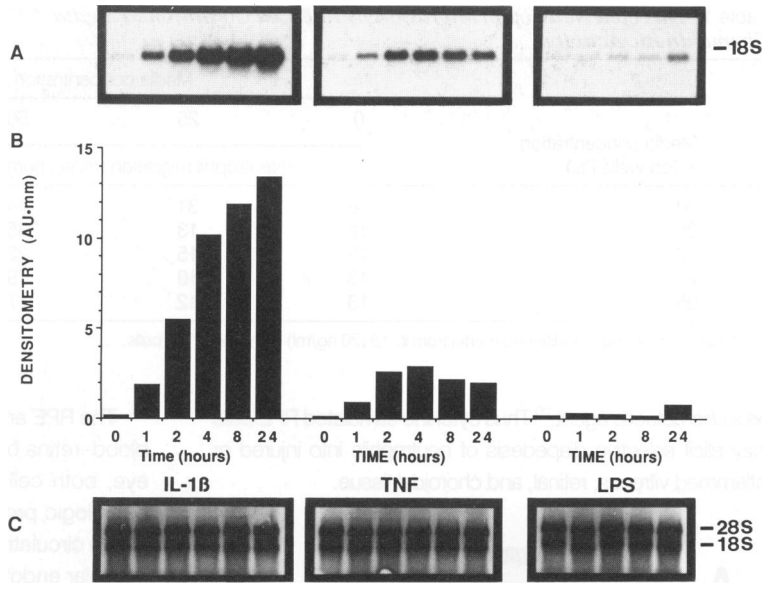

Figure 1. *Time-dependent induction of RPE NCF mRNA expression by IL-1β, TNF-α, and LPS. Human RPE cells were exposed to IL-1β (20 ng/ml), TNF-α (20ng/ml), or LPS (10μg/ml) at 37° C for the times indicated. The medias were removed and total cellular RNA was extracted and evaluated by Northern blot analysis for NCF mRNA.* **A:** *Twelve-hour autoradiograph of Northern blot analysis of NCF mRNA from RPE cells treated with either IL-1β, TNF-α, or LPS, respectively;* **B:** *relative density of NCF mRNA signals determined by laser densitometry;* **C:** *18 and 28 s rRNA of the same blots. NCF mRNA was not detected in unstimulated RPE cultures assayed for 24 hours.*

hours after IL-1β challenge showed progressive increases over the range of 0.02 to 20 ng/ml (data not shown). Maximal neutrophil chemotaxis was found at 20 ng/ml and 50% of the maximal activity occurred at 0.2 ng/ml.

Expression of 28 and 18 s rRNA was not altered by IL-1β, TNF-α, or LPS in any of the experiments (Figures 1 and 3). Retinal pigment epithelium cell NCF mRNA migration on the Northern blots (1.8 kb) was identical to that reported for monocyte-derived NCF.[8] Supernatants from unstimulated RPE cells had no NCF activity. Media alone or media containing IL-1β, TNF-α, or LPS also failed to demonstrate significant NCF activity.

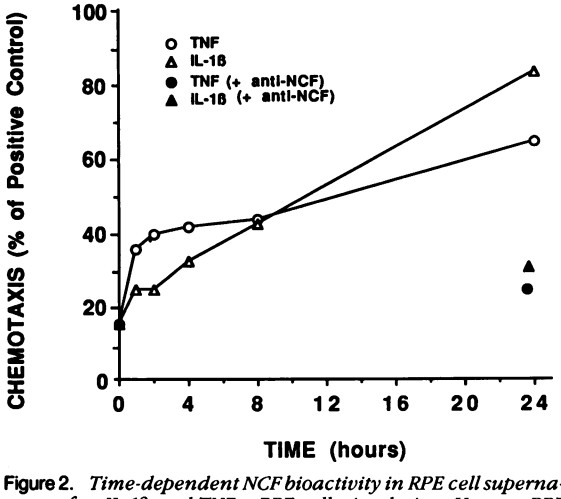

Figure 2. *Time-dependent NCF bioactivity in RPE cell supernatants after IL-1β and TNF-α RPE cell stimulation. Human RPE cells were exposed to IL-1β (20 ng/ml) or TNF-α (20 ng/ml) at 37 C for the times indicated. The medias were removed and their neutrophil chemotactic activities were measured using modified Boyden chambers, as described in Materials and Methods. Chemotaxis is expressed as percentage of positive f-MLP control. Preincubating aliquots with anti-human rNCF antibody blocked the bioactivity of the RPE supernatants by 60%. Preincubation with preimmune serum did not block any chemotactic activity in parallel assays. Chemotactic activity was not detected in media from unstimulated RPE cultures.*

Discussion

NCF is a 72-amino acid polypeptide that binds specific receptors on neutrophils, thereby initiating Ca^{++}-dependent and *Bordetella pertussis* toxin-inhibitable chemotaxis, degranulation, and respiratory burst.[16] Although initially isolated from stimulated monocytes,[17] tissue-based vascular endothelial cells[18] and fibroblasts[15] also have been shown to produce NCF after stimulation with endotoxin, IL-1β, and TNF-α. The vascular leakage and other tissue effects induced by NCF (IL-8) appear to result from local neutrophil activation.[16]

Retinal pigment epithelium cell alterations have been implicated as central components of several common eye diseases.[6] Inflammatory cells, vascular endothelial cells, fibroblasts, and astrocytes also actively participate in these blinding eye diseases and are known producers of IL-1β and TNF-α. Nevertheless, the responses of retinal cells to cytokines, other than the interferon-γ–enhanced expression of RPE histocompatibility antigens,[19] have not been investigated. Our results show that RPE cells exposed to 0.2 ng/ml (60 U/ml) of IL-1β produce steady-state NCF mRNA levels similar to those found in mononuclear phagocytes stimulated with 100 U/ml of IL-1.[8] Our checkerboard analysis further indicates that NCF is a true chemotactic factor with minimum chemokinetic activity, corroborating previously published results demonstrating NCF to

Table 1. *Directed Neutrophil Migration by RPE Cell Conditioned Media: * Chemotactic Activity Distinguished from Chemokinetic Activity*

Media concentration in top wells (%)	Media concentration in bottom wells (%)				
	0	25	50	75	95
	Neutrophil migration (mean number in 10 high powered fields)				
0	8	31	44	58	90
25	12	13	35	35	63
50	13	15	22	38	50
75	13	10	19	19	39
95	13	12	17	20	23

* Twenty-four-hour conditioned media from IL-1β (20 ng/ml)-stimulated RPE cells.

be a chemotactic agent.[20] Thus cytokine-stimulated RPE cells may elicit selective diapedesis of neutrophils into injured or inflammed vitreous, retinal, and choroidal tissue.

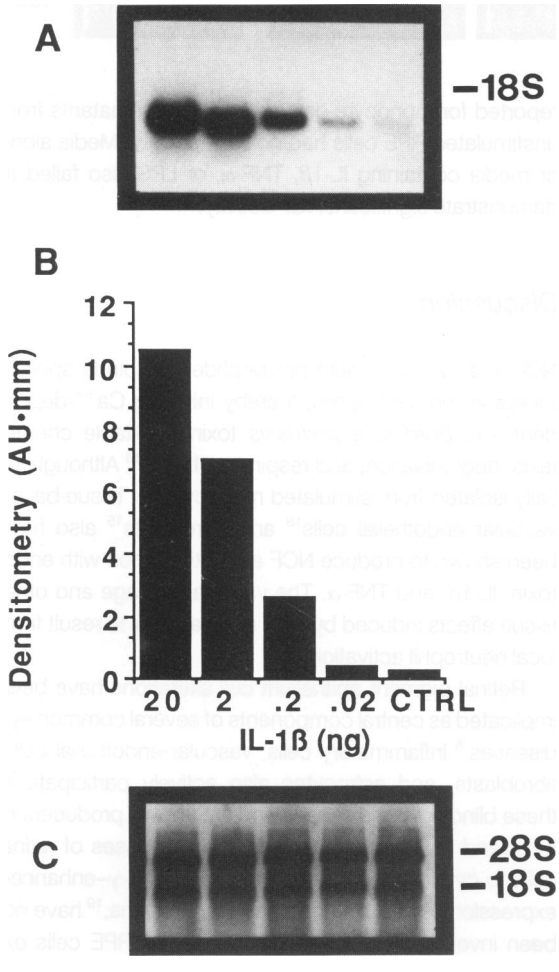

Figure 3. *Progressive NCF mRNA induction by increasing concentrations of IL-1β. Human RPE cells were exposed to the IL-1β concentrations indicated for 4 hours at 37 C. The medias were removed and total cellular RNA was extracted and evaluated by Northern blot analysis for NCF mRNA.* A: *Northern blot analysis of mRNA from cells stimulated with increasing doses of IL-1β and probed for NCF mRNA;* B: *relative density of the signals determined by laser densitometry;* C: *18 and 28 s RNA of the same blots.*

The RPE and the retinal vascular endothelium form the blood–retina barrier.[1] Due to their strategic position in the eye, both cell types actively participate in many retinal pathologic processes and influence the interactions between circulating leukocytes and the retina.[6] The RPE and vascular endothelial cells share several features that may be important in their physiologic and pathologic roles. For example, TNF-stimulated vascular endothelial cells express surface histocompatability antigens[21] and neutrophil adherence proteins[22,23] and produce biologically active arachidonic acid metabolites,[24] tissue factor,[25] IL-1,[26,27] and platelet-derived growth factor.[28] Retinal pigment epithelium cells also express histocompatability antigens,[19] contain high levels of arachidonic acid,[29] produce and respond to growth factors,[30] and induce vascular endothelial procoagulant activity.[31] Our results extend these similarities and show that RPE cells, as well as vascular endothelial cells, produce NCF indistinguishable from monocyte-derived NCF when stimulated with IL-1β, TNF-α, or LPS. The elaboration of NCF by vascular endothelial and RPE cells, which constitute the blood–retina barrier, emphasizes that these critically positioned, tissue-based cells are not passive targets for injury, but active participants in ocular disease.

Retinal pigment epithelium- and vascular endothelial-derived NCF may play a central role in experimental uveitis, which is induced by IL-1β, TNF-α, or LPS,[32] and experimental lens-induced uveitis in which high choroidal levels of IL-1β and TNF have been found.[33] Furthermore, neutrophil chemotactic activity recently has been reported in vitreous samples from patients with severe diabetic retinopathy or proliferative vitreoretinopathy, which is the principal cause of failure of retinal detachment repair.[34] Identification of RPE-derived cytokines such as NCF (IL-8) illuminates important mechanisms through which resident, neural-derived phagocytic RPE cells may participate in common and blinding ocular diseases.

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