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TRANSFORMING GROWTH FACTOR-ß INHIBITS THE CYTOKINE-MEDIATED EXPRESSION OF THE INDUCIBLE NITRIC OXIDE SYNTHASE mRNA IN HUMAN RETINAL PIGMENT EPITHELIAL CELLS

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SUMMARY: Human retinal pigment epithelial (RPE) cells in culture respond to a mixture of cytokines (IFN-γ, IL-1ß, TNF-α) by producing large amounts of nitric oxide. Transforming growth factor-ß, unlike other growth factors, was found to inhibit this response by more than 75%. The expression of mRNA for the inducible form of nitric oxide synthase in RPE cells treated with cytokines was demonstrated by reverse transcription-polymerase chain reaction, sequencing of the PCR product and northern blotting. Transforming growth factor-ß was highly effective in inhibiting (by 75%) the cytokine-induced nitric oxide synthase mRNA expression. This response by RPE may play an important role in the etiology of infectious and inflammatory diseases affecting retina.

Nitric oxide (NO) is cytotoxic and microbiostatic, and its increased production is indicated in pathophysiological conditions associated with several vascular, inflammatory and neurodegenerative disorders (recently reviewed in 1). Generation of NO in large amounts is attributed to the inducible form of nitric oxide synthase (iNOS), which is highly induced in many cell types by inflammatory cytokines and endotoxins (2-6). There are indications that NO is involved in the etiology of certain ocular inflammatory disorders (7). Retinal pigment epithelium (RPE), a single layer of epithelial cells localized between choroid and neuroretina in the eye, plays a critical role in infectious and inflammatory diseases associated with retina (8). Indeed, retinal pigment epithelial (RPE) cell cultures from bovine, mouse and rat have been found to respond to cytokines by increasing the production of NO (9-11). RPE cells of human origin also respond to cytokines by increasing the production of NO (12). However, little is known as to the expression of iNOS and its regulation by growth factors at the molecular level. The present study shows that human RPE cells respond to cytokines by

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increasing the expression of iNOS mRNA. Also TGF-ß, unlike other growth factors tested, inhibits NO production by blocking the cytokine-induced expression of iNOS mRNA.

MATERIALS AND METHODS

Human RPE cells: Human RPE cell cultures were established from explants derived from human donor eyes as previously described (13,14). Minimum essential medium supplemented with fetal bovine serum (10%), non-essential amino acids, penicillin (100 u/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) was used as the growth medium. The RPE cells in culture exhibited typical epithelial cell morphology and tested positive when immunostained with a monoclonal antibody against cytokeratin (13). Cells from passages 6 to 12 were used in this study.

NO production by RPE cells: The cells were grown to confluency on 24 well plates and the growth medium was replaced with the incubation medium (growth medium devoid of both fetal bovine serum and phenol red, 0.7 ml/well) containing cytokines and/or other agents. The cells were incubated for the specified time interval and nitrite concentration in the medium, which reflects NO production by the cells, was estimated using Griess reagent (5,15). Briefly, 100 μl of medium was mixed with 100 μl of Griess reagent (a 1:1 mixture of a 1% solution of sulfanilamide in 5 % phosphoric acid and a 0.1% aqueous solution of naphthylethylenediamine dihydrochloride) and the color developed was estimated spectrophotometrically at 540 nm. Sodium nitrite was used as the standard.

Reverse transcriptase-polymerase chain reaction (RT-PCR): RNA preparations were isolated from human RPE cells treated with cytokines, reverse transcribed using an oligo dT primer, and the first strand cDNA preparations were used as templates for PCR (16) as described before (17). Three sets of primers: (a) 5'- TCC AAC CTG CAG GTC TTC GAT and 5'- TTC CTC CAG GAT GTT GTA GCG, (b) 5'- CGC TAC AAC ATC CTG GAG GAA and 5'- TCC ATG ATG GTC ACA TTC TGC and (c) 5'- TCC AAC CTG CAG GTC TTC GAT and 5'- GAA GTC GTG CTT GCC ATC ACT were designed from the cDNA sequence reported for human hepatocyte iNOS (2) to amplify 825-1388, 1368-1510, and 825-977 bp regions, respectively. The oligodeoxynucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer and purified using oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA). Primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Clontech. A reaction mixture (50 μl) consisting of the cDNA preparation (corresponding to 100 ng of RNA), primers (0.5 μM each), deoxynucleoside triphosphates (200 μM), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatin, and Taq DNA polymerase (2.5 units) was heated at 95°C for 2 min and subjected to 35 cycles at 94^0 C for 30 sec, 58^0 C for 1 min, and 72^0 C for 1 min. The reaction mixture was kept at 72^0 C for 10 min. An aliquot (20 μl) was analyzed for amplification product by Agarose gel electrophoresis followed by ethidium bromide staining.

Northern blot analysis: The total RNA fraction isolated from treated cells was subjected to agarose gel electrophoresis in the presence of formaldehyde. The RNA from the gel was capillary blotted onto an Immobilon N membrane (Millipore Corporation, Bedford, MA), UV cross-linked and hybridized using the inducible NO synthase cDNA probe under conditions described before (14). A PCR product corresponding to the 825-1388 bp region of cDNA for hepatocyte iNOS (2) was used as the probe after labeling with ^{32}P by random priming (18), and hybridization was carried out at 65°C. The blots were then washed under stringent conditions and exposed to Kodak X-OMAT AR film.

DNA sequencing: The PCR product prepared with primer set "a" was electrophoretically separated on an agarose gel, extracted using a gel extraction kit (Qiagen, Chatsworth, CA), and subjected to automated DNA sequencing (370A DNA sequencer, Applied Biosystems).

RESULTS

TNF-α, IFN-γ, IL-1β and lipopolysaccharide (LPS) were tested for their ability to induce NO production by human RPE cells. None of these agents were effective individually. A combination of IFN-γ and IL-1β was found to be the minimum requirement for appreciable increase in NO production (Fig. 1). More than a 20 -fold increase over the control value was observed in this case. TNF-α, but not LPS, showed a moderate increase in NO production when combined with IFN-γ and IL-1β. Therefore, the combination of TNF-α, IFN-γ and IL-1β , hereafter referred to as "cytokine mixture", was used in further experiments. Nitric oxide synthase inhibitors L-N^{ω}-nitro-L-arginine (0.1 mM) and L-N^{ω}-nitro-L-arginine methyl ester (0.1 mM) inhibited the "cytokine mixture"- induced NO production by 50 and 58%, respectively (data not shown).

The NO production, estimated as nitrite concentration in the culture medium, in RPE cells exposed to "cytokine mixture" was time dependent (Fig. 2). The NO production by RPE cells at the end of 12, 24, 48, and 72 h of incubation were about 5, 15, 70, and 85 nmol/10^6 cells, respectively. In comparison, medium from control cells did not show the presence of

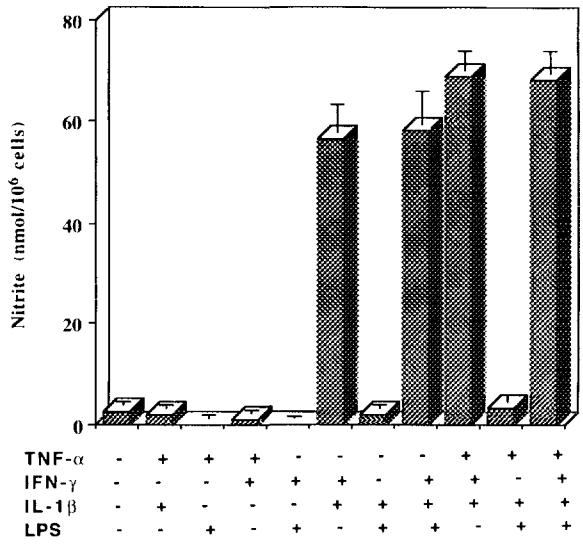

Figure 1. Effects of TNF-α, IFN-γ, IL-1β and LPS on NO production by human RPE cells. Nitrite was estimated in the culture medium after cells were incubated for 72 h with indicated combination of TNF-α (10 ng/ml), IFN-γ (100 u/ml), IL-1β (10 ng/ml) and LPS (10 μg/ml). Results are means ± SE for four experiments.

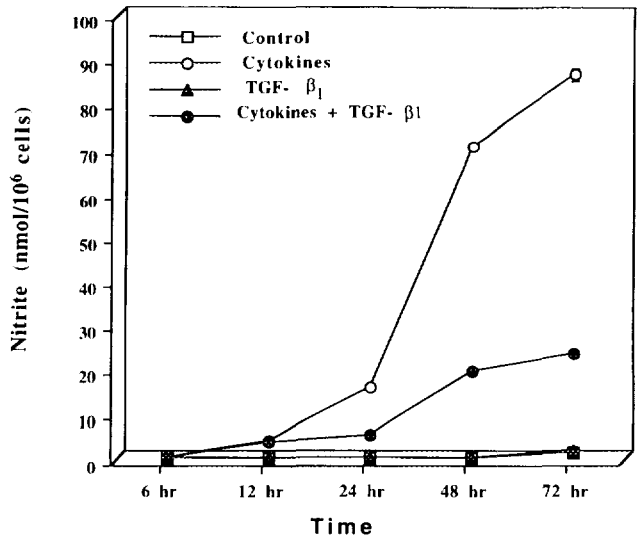

Figure 2. Time course of NO production by human RPE cells treated with cytokines and TGF-ß. The cells were incubated with or without cytokine mixture, TNF-α (10 ng/ml), IFN-γ (100 u/ml) and IL-1ß (10 ng/ml), in the presence or absence of TGF-ß (10 ng/ml). The media were taken at the indicated time interval for nitrite estimation. Results are means ± SE for four experiments.

detectable amount of nitrite. TGF-ß1 was highly effective in inhibiting the nitrite accumulation in the medium of treated cells (Figs. 2 and 3). The inhibition by TGF-ß1 was about 70% at both 48 and 72 h. TGF-ß2, when tested, also exhibited similar inhibition (data not shown). In contrast, other growth factors like TGF-α, PDGF-AB, EGF, IGF-1 and bFGF showed either no effect or a slight increase in nitrite production by RPE cells exposed to cytokine mixture (Fig. 3). Dexamethasone, reported to be an inhibitor of cytokine-mediated NO production in other cell types (19), was not effective in RPE cells.

Human RPE cells were tested for their ability to express the mRNA for the inducible form of nitric oxide synthase. An RT-PCR method was developed for this purpose. RNA preparations obtained from cells exposed to "cytokine mixture" were reverse transcribed and subjected to PCR using three sets of primers (see MATERIALS AND METHODS). Samples were then analyzed by agarose gel electrophoresis. Ethidium bromide staining of the gels showed the presence of amplification products of expected sizes in all three cases (Fig. 4). When the PCR product obtained with primer set "a" was gel purified and analyzed by DNA sequencing, it showed 100% homology to the 848-1350 bp region of the cDNA sequence reported by Geller et al. (2) for human hepatocyte iNOS (data not shown). Northern blot analysis was employed to obtain more information about mRNA for inducible iNOS expressed in RPE cells (Fig. 5). The signal was not detectable in control cells. However, a

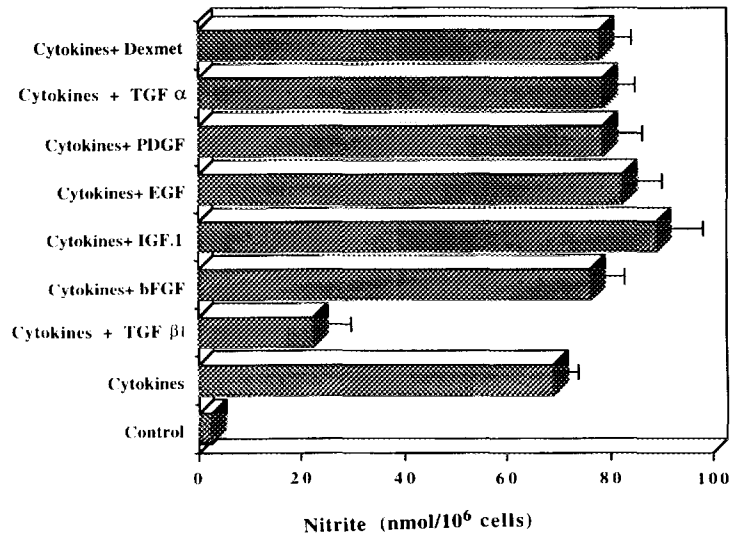

Figure 3. Effect of growth factors and dexamethasone on NO production in human RPE cells treated with cytokines. Nitrite was estimated in the media after the cells were incubated for 72 h with TNF-α (10 ng/ml), IFN-γ (100 u/ml) and IL-1ß (10 ng/ml) and the indicated agent: TGF-ß1 (10 ng/ml), bFGF (20 ng/ml), IGF-1 (100 ng/ml), EGF (1μg/ml), PDGF-AB (100 ng/ml), TGF-α (1 μg/ml) or dexamethasone (10 μM). Results are means ± SE for four experiments.

4.5 kb signal, similar to that reported for hepatocytes (2), was detected in RPE cells exposed to "cytokine mixture".

The effect of various agents on the expression of the mRNA for iNOS in RPE cells was examined by RT-PCR (Fig. 6). The PCR (25 cycles) was performed in the presence of trace amounts of [α-^{32}P]dCTP and the radioactivity incorporated into the band corresponding to the amplification product was estimated. PCR was also done in the presence of primers for GAPDH as a control. The radioactivity associated with iNOS band was extremely low in untreated cells. Incubation with a mixture of IFN-γ and IL-1ß increased the radioactivity incorporated into the iNOS band by about 27-fold. Addition of TNF-α to this mixture showed a further increase in the radioactivity incorporated (to about 45-fold). TGF-ß was highly effective in blocking the effect mediated by the "cytokine mixture". In contrast, bFGF showed no effect.

DISCUSSION

Human RPE cells respond to inflammatory cytokines by increasing the production of NO. The combined presence of IFN-γ and IL-1ß was found to be required for a good response. Inclusion of TNF-α further enhanced their effect. Human RPE cells, unlike other

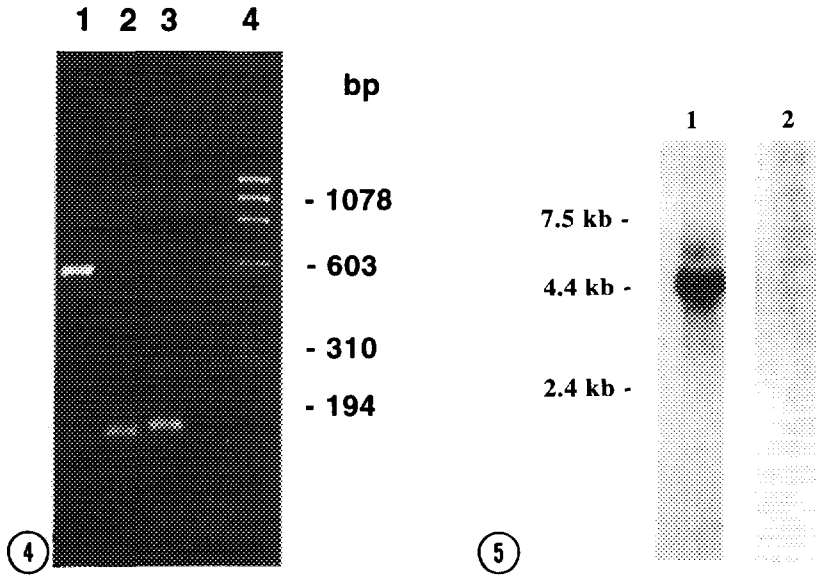

Figure 4. RT-PCR analysis of inducible NO synthase mRNA expression in human RPE cells. RNA preparation from the cells treated for 24 h with TNF-α (10 ng/ml), IFN-γ (100 u/ml) and IL-1ß (10 ng/ml) was reverse transcribed and analyzed by PCR. The products were separated by Agarose gel electrophoresis and visualized by ethidium bromide staining. Lanes 1,2 and 3 show samples from PCR with primer sets "a", "b" and "c", respectively. Lane 4 shows DNA size markers.

Figure 5. Northern blot analysis of inducible NO synthase expression in human RPE cells. RNA preparations from the cells incubated for 24 h with or without a mixture of TNF-α (10 ng/ml), IFN-γ (100 u/ml) and IL-1ß (10 ng/ml) was analyzed by northern blotting using iNOS cDNA probe. 1, treated; 2, control.

cell types like human hepatocytes (2), human chondrocytes (4), bovine RPE (9), and mouse RPE (10), do not show any response to LPS. The increase of NO production in human RPE cells was preceded by an increase in the expression of a 4.5 kb iNOS mRNA similar to that of human hepatocytes (2). RT-PCR analysis also showed that the mRNA expressed in RPE cells is similar to that characterized from hepatocytes (2). In agreement, an RT-PCR product when sequenced showed 100% homology to the 848-1350 bp region of hepatocyte iNOS cDNA.

TGF-ß, as previously reported (12), was found to markedly inhibit NO production by human RPE cells treated with the cytokines. We have also shown that this effect was unique to TGF-ß, since a number of other growth factors failed to show this inhibitory effect. TGF-ß has been reported to block the cytokine-mediated increase in NO production in a variety of cells (6,10,12,20). Growth factors such as EGF (6), PDGF (21) and FGF (9) have also been shown to inhibit cytokine-mediated NO production in some cell types. It is interesting to note that NO production by bovine RPE cells exposed to cytokines is stimulated by TGF-ß and

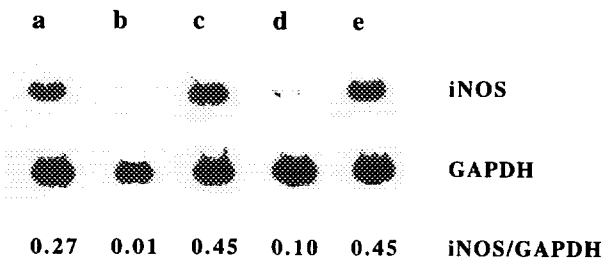

Figure 6. Effect of TGF-ß1 and bFGF on inducible NO synthase mRNA expression in human RPE cells treated with cytokines. RNA extracted from cells following 24 h of treatment, reverse transcribed and subjected to PCR (28 cycles) using primer set "a". The reaction mixture (50 µl) also contained 1 µCi of [α-^{32}P]dCTP (specific activity 3000 Ci/mmol). Similar PCR conditions were employed for GAPDH primers except that the number of cycles was reduced to 19. Samples were then subjected to polyacrylamide gel electrophoresis. Gels were dried and scanned using a radioanalytic detector to estimate the radioactivity incorporated into amplification products. a, IFN-γ (100 u/ml) and IL-1ß (10 ng/ml); b, control; c, TNF-α (10 ng/ml), IFN-γ (100 u/ml) and IL-1ß (10 ng/ml); d, TNF-α (10 ng/ml), IFN-γ (100 u/ml), IL-1ß (10 ng/ml) and TGF-ß (10 ng/ml); and e, TNF-α (10 ng/ml), IFN-γ (100 u/ml), IL-1ß (10 ng/ml) and bFGF (20 ng/ml).

inhibited by bFGF (9). Our present study shows that, in human RPE cells, TGF-ß elicits its response by effectively blocking the increase in the expression of iNOS mRNA. The mechanism of this effect remains to be elucidated. In summary, therefore, we have found that the production of NO by RPE cells in response to cytokines and its inhibition by TGF-ß is modulated at the level of iNOS mRNA expression. Since TGF-ß is implicated in retinal disorders such as proliferative vitreoretinopathy (22), this response may play an important role in the pathophysiology of ocular diseases.

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