Hypoxia-Stimulated Vascular Endothelial Growth Factor Production in Human Nasal Polyp Fibroblasts

Effect of Epigallocatechin-3-Gallate on Hypoxia-Inducible Factor-1α Synthesis

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**Objective:** To verify the inhibitory effects of epigallocatechin-3-gallate (EGCG) on the synthesis of hypoxia-induced vascular endothelial growth factor (VEGF) in nasal polyp fibroblasts (NPFs).

**Design:** Eight primary cultures of NPFs were established from nasal polyps. Effects of EGCG on the production of hypoxia-inducible factor (HIF)-1α (the most potent VEGF stimulant) and VEGF by NPFs under hypoxic conditions were measured by Western blot analysis. Immunohistochemical staining was used to examine the in vivo expressions of HIF-1α and VEGF in 20 sections of nasal polyps.

**Results:** Western blot analysis showed that cobalt chloride induced HIF-1α and VEGF synthesis in NPFs in a time-dependent manner, reaching a plateau at 4 and 8 hours, respectively, following treatment. Epigallocatechin-3-gallate attenuated the level of HIF-1α induced by cobalt chloride and also reduced cobalt chloride-stimulated VEGF production by suppressing HIF-1α synthesis. Furthermore, oligomycin (a specific HIF-1α inhibitor) combined with EGCG resulted in a more profound inhibition of VEGF synthesis compared with oligomycin or EGCG treatment alone. Nevertheless, the synergistic effect seemed smaller than the sum of their individual actions. Immunohistochemical analysis revealed the presence of HIF-1α and VEGF in NPFs and mononuclear round cells. Intimate alignment of VEGF-positive fibroblasts and proliferating small capillaries was frequently found.

**Conclusions:** Nasal polyp fibroblasts contribute to the pathogenesis of nasal polyps by producing VEGF to promote angiogenesis under hypoxic conditions. Epigallocatechin-3-gallate substantially diminishes HIF-1α and VEGF synthesis in NPFs.

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**NASAL POLYPS ARE THE** most commonly found mass lesions in the nasal cavity.1 Although the pathogenesis remains unclear, many authors believe nasal polyps to be an inflammatory disorder.2 We recently found a connection between nasal polyposis and infiltration of macrophages, an indispensable element in inflammation.3 We have also demonstrated active synthesis of essential proinflammatory mediators such as interleukin 6 and cyclooxygenase-2 in tumor necrosis factor α-stimulated nasal polypl fibroblasts (NPFs), substantiating the importance of stromal cells in nasal polyposis.4

Angiogenesis, the process by which new vascular networks develop from capillary buds or sprouts sent out by preexisting vessels, has an essential role in the progression of tumor5 and many chronic diseases such as rheumatoid arthritis.6 The angiogenic process is precisely modulated by a vast array of proangiogenic (to increase blood vessels when needed) and antiangiogenic (to block pathologic angiogenesis) factors. Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors, capable of promoting proliferation and migration of endothelial cells and increasing vascular permeability.7 By demonstrating increased levels of VEGF in cultured human NPFs, Matsune et al8 and Sun et al9 proposed the role of VEGF in the pathogenesis of nasal polyps.

The stimuli for VEGF expression derive primarily from hypoxia, which activates VEGF by increasing hypoxia-inducible factor-1 (HIF-1),10 a heterodimeric transcriptional factor consisting of α and β subunits. The availability of HIF-1 is determined primarily by HIF-1α, which is regulated in an oxygen-sensitive manner by degradation through the ubiquitin-proteasome pathway under normoxia, in contrast to the stably expressed HIF-1β subunit. With hypoxia, HIF-1α is stabilized, translocated to the nucleus, heterodimerizes with HIF-1β, and activates...
multiple genes involved in angiogenesis and other cellular functions.

Green tea is made by brewing the leaves of *Camellia sinensis* to inactivate the polyphenol oxidase. Polyphenols are the major constituents of brewed green tea, of which epigallocatechin-3-gallate (EGCG) is the most abundant and active. The antioxidant and free radical scavenger properties of EGCG are believed to be primarily responsible for the protective effect of tea consumption against the risks of cancer, neuron degeneration, and coronary artery diseases. An inhibitory effect of EGCG on VEGF production and proliferation has been reported in several cancer cell lines, however, a similar action of EGCG has never been demonstrated in NPFs. This deserves further investigation because it may lead to the development of a new therapeutic strategy for nasal polyps. In this study, we demonstrated that EGCG inhibits cobalt chloride (CoCl₂)-induced VEGF synthesis in NPFs, possibly by diminishing HIF-1α synthesis.

**TISSUE SAMPLES**

Nasal polyps, mainly originating from the ethmoidal labyrinth and present in the middle meatuses, were obtained at functional endoscopic sinus surgery with the Messerklinger-Stammberger modification for treatment of bilateral chronic sinusitis with sinonasal polyposis. Patients with a single polyp (antrochoanal polyp) or with other diseases related to nasal polyps such as cystic fibrosis, primary ciliary dyskinesia, and fungal sinusitis were excluded from this study. Bilateral chronic sinusitis with sinonasal polyposis was diagnosed on the basis of history and findings at clinical examination, nasal endoscopy, and sinus computed tomography. No patients had a history of nasal allergy, asthma, or aspirin hypersensitivity. All had normal serum antigen-specific IgE antibody (Opticon multiple allergen-specific IgE assay; Hitachi Diagnostics, Inc, Mountain View, California), and none had used regular topical or oral medication within 3 weeks. Informed consent was obtained before surgery. The study was approved by the ethics committee of National Taiwan University Hospital, Taipei, Taiwan.

**CELL CULTURES**

Eight primary cultures of NPFs were established from nasal polyps from 8 patients, respectively, as previously described. In brief, after removing the epithelial layer, the specimens were immersed overnight in Dulbecco modified Eagle medium (calcium chloride, 265 µg/mL; iron nitrate, 0.1 µg/mL; potassium chloride, 400 µg/mL; magnesium sulfate, 200 µg/mL; sodium chloride, 6400 µg/mL; sodium bicarbonate, 700 µg/mL; sodium phosphate, 125 µg/mL; phenol red, 15 µg/mL; HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid] buffer [pH 7.4], 25 mmol/L, and D-glucose, 1 mg/mL) containing 10% fetal calf serum and antibiotics (penicillin, 100 U/mL; streptomycin, 100 µg/mL; and amphotericin B, 0.5 µg/mL). The samples were minced into 1-mm³ fragments and covered with sterilized glass coverslips. After the fibroblasts migrated from tissue explants and became confluent, the cells were trypsinized and subcultured. Cells between passages 3 to 6 were plated at a density of 5 × 10⁵/mL on 10-cm dishes and subjected to various stimuli. Before different treatments, the cells were made quiescent in serum-free media (media without fetal calf serum) for 24 hours. Data given herein are the means of 8 experiments.

**METHODS**

**WESTERN BLOT ANALYSIS**

Western blot analysis was performed as previously described. In brief, cells were lysed in lysis buffer, fractionated by sodium dodecylsulfate–polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Antibodies against VEGF (R&D Systems, Inc, Minneapolis, Minnesota) and HIF-1α (Upstate Biotechnology Inc, Lake Placid, New York) were added at concentrations suggested by the manufacturer. Proteins were visualized with horseradish peroxidase–conjugated immunoglobulin and an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, New Jersey). The membranes were reprobed with β-actin antibody after washing with stripping buffer.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Sections were collected from the pedicle area of an entire nasal polyp in each of 20 patients, including the 8 patients whose nasal polyps were used for primary cell culture. Sections cut from 10% formalin-fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated, and subjected to antigen retrieval using the microwave method. After blocking endogenous peroxidase with hydrogen peroxide and nonspecific binding with rabbit serum in TRIS-buffered saline solution, the sections were incubated with antibodies against human VEGF (dilution 1:40; R&D Systems, Inc) or human HIF-1α (dilution 1:50; Novus

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Biologics, Inc, Littleton, Colorado) in TRIS-buffered saline solution overnight. Sections were incubated with a biotinylated secondary antibody and colorization using diaminobenzidine or 3-amino-9-ethylcarbazole. Counterstaining was performed using Mayer hematoxylin for 1 minute.

STATISTICAL ANALYSIS

Data were subjected to analysis of variance for multiple comparisons and then the Fisher protected least significant difference test. \( P < .05 \) was considered statistically significant.

RESULTS

CoCl\(_2\) STIMULATED VEGF SYNTHESIS IN NPFs

The amounts of VEGF produced in NPFs stimulated by CoCl\(_2\) (500 \( \mu \)mol/L) during various incubation periods were assessed by Western blot analysis. The results showed that CoCl\(_2\) induced VEGF synthesis in NPFs. The increase in levels of VEGF were time dependent, reaching a peak at 8 hours following treatment (Figure 1).

EGCG REDUCED THE LEVELS OF CoCl\(_2\)-STIMULATED HIF-1\( \alpha \)

Cells were incubated with CoCl\(_2\) (500 \( \mu \)mol/L) for various incubation periods, and HIF-1\( \alpha \) levels were determined by Western blot analysis. Western blot analysis revealed that the peak level of HIF-1\( \alpha \) in NPFs treated with CoCl\(_2\) occurred at 4 hours and declined thereafter (Figure 2A and C). When NPFs were incubated with CoCl\(_2\), alone or in combination with EGCG (10 \( \mu \)g/mL) (3 hours before the addition of CoCl\(_2\)) for 4 hours, HIF-1\( \alpha \) levels were analyzed. Epigallocatechin-3-gallate significantly diminished CoCl\(_2\)-induced HIF-1\( \alpha \) synthesis (Figure 2B and D).

EGCG ABROGATED CoCl\(_2\)-STIMULATED VEGF PRODUCTION BY REDUCING HIF-1\( \alpha \) SYNTHESIS

To verify the involvement of HIF-1\( \alpha \) in the synthesis of VEGF following CoCl\(_2\) stimulation, oligomycin, a specific inhibitor of HIF-1\( \alpha \),\(^{18}\) was applied in the subsequent experiment. Nasal polyp fibroblasts were incubated with CoCl\(_2\), alone or in combination with EGCG (10 \( \mu \)g/mL)
or oligomycin (10 µg/mL) (3 hours before the addition of CoCl2) for 4 hours, and HIF-1α levels were assessed by Western blot analysis. The results showed that oligomycin attenuated the level of CoCl2-stimulated VEGF significantly. Epigallocatechin-3-gallate also diminished the level of VEGF induced by CoCl2 (Figure 3). Compared with treatment with oligomycin or EGCG alone, more pronounced inhibition of VEGF synthesis was obtained when oligomycin and EGCG were administered simultaneously. However, the synergism was incomplete, inasmuch as the combination effect seemed smaller than the sum of their individual action (Figure 3).

**IMMUNOLOCALIZATION OF HIF-1α AND VEGF IN NASAL POLyps**

At microscopy, the presence of HIF-1α was detected in subepithelial fibroblasts and mononuclear round cells (Figure 4A and C). Fibroblasts usually are spindle shaped with an ovoid nucleus with no prominent nucleolus, and the immunohistochemical stain around the HIF-1α is located in the cytoplasm of the fibroblasts. The mononuclear round cells include macrophages, lymphocytes, and plasma cells. Differentiation of those cells is in their structure: macrophages usually have abundant cytoplasm and are the largest of these cells, lymphocytes have scanty cytoplasm, and plasma cells have an eccentric nucleus and are relatively the same size as lymphocytes. Vascular endothelial growth factor was located in some basal cells of the lining epithelium, plump endothelial cells lining the proliferating capillaries, mononuclear round cells, and fibroblasts. Intimate alignment of long, spindle-shaped, VEGF-positive fibroblasts and proliferating small capillaries was frequently found (Figure 4B and D).

**COMMENT**

By promoting proliferation and migration of endothelial cells and increasing vascular permeability, VEGF has an essential role in mediating angiogenesis in normal and pathologic conditions such as embryonic development, wound healing, and tumor growth.5-7 Vascular endothelial growth factor is also involved in chronic inflammatory and immunologic processes, leading subsequently to the formation of nasal polyps.8,9 The results of our study showed that CoCl2 induced VEGF synthesis in NPFs. In this experimental model, CoCl2 was used to initiate a hypoxic culture condition. Treatment of cultured cells with CoCl2, alone or in combination with EGCG (10 µg/mL) or oligomycin (10 µg/mL) (3 hours before the addition of CoCl2) for 4 hours, and HIF-1α levels were assessed by Western blot analysis. B, Results were quantified by densitometric analysis, normalized by the level of β-actin, and expressed as fold change relative to untreated control.

Western blot analysis revealed that CoCl2 stimulated HIF-1α production in NPFs, and immunohistochemical staining also showed the in vivo presence of HIF-1α in NPFs and mononuclear round cells, implying that nasal polyps are in hypoxia. To our knowledge, this is the first demonstration of HIF-1α synthesis in NPFs. In hypoxia HIF-1α translocates to the nucleus, heterodimerizes with HIF-1β and activates multiple genes including VEGF.10,11 Our results demonstrate that oligomycin, a specific inhibitor of HIF-1α, attenuated the level of CoCl2-stimulated VEGF production. By using oligomycin as an inhibitor of HIF-1α, the role of HIF-1α in CoCl2-stimulated VEGF production in NPFs was confirmed. The influence of EGCG on hypoxia-induced VEGF and HIF-1α production was examined subsequently, and suppressive effects on both

**Figure 3.** Epigallocatechin-3-gallate (EGCG) reduced cobalt chloride (CoCl2)-stimulated vascular endothelial growth factor (VEGF) production by inhibiting hypoxia-inducible factor (HIF-1α) synthesis. A, Nasal polyp fibroblasts were incubated with CoCl2, alone or in combination with EGCG (10 µg/mL) or oligomycin (10 µg/mL) (3 hours before the addition of CoCl2) for 4 hours, and HIF-1α levels were assessed by Western blot analysis. B, Results were quantified by densitometric analysis, normalized by the level of β-actin, and expressed as fold change relative to untreated control.

*P<.05 vs control. †P<.05 vs CoCl2 at 500 µmol/L. Each bar represents the mean (SD) of 8 experiments. Note that EGCG and oligomycin attenuated the level of CoCl2-stimulated VEGF significantly. A more profound inhibition was noted when EGCG and oligomycin were administrated together.
molecules were found. Of the mechanisms on the alleviation of EGCG on CoCl₂-stimulated VEGF synthesis, one explanation is that EGCG may inhibit VEGF production by downregulating HIF-1α or possibly by affecting the effect of CoCl₂ on cell culture in creating or mediating the hypoxic effect. Inasmuch as non–HIF-1α pathways such as activator protein–1 and nuclear factor–kappa B are reportedly involved in VEGF expression, another scenario is inhibition of EGCG on hypoxia-induced VEGF synthesis by changing the effects of activator protein–1 or nuclear factor–kappa B in addition to downregulating the levels of HIF-1α. The proposal deserves further investigation because the results may, to a certain degree, account for our finding that the combined effect of EGCG and oligomycin on VEGF production was higher than that induced by either agent alone.

In conclusion, our data suggest that NPFs may contribute to the pathogenesis of nasal polyps by producing VEGF to promote angiogenesis under hypoxia. Epigallocatechin-3-gallate diminishes HIF-1α and VEGF synthesis substantially in cultured NPFs. However, real physical conditions are far more complicated than the in vitro culture system. Therefore, further clinical studies are needed to verify the feasibility of EGCG as a therapeutic agent for nasal polyposis.

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