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.
multiple genes involved in angiogenesis and other cellular functions.\textsuperscript{11}

Green tea is made by brewing the leaves of \textit{Camellia} \textit{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.\textsuperscript{12} 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.\textsuperscript{13,14} An inhibitory effect of EGCG on VEGF production has been reported in several cancer cell lines\textsuperscript{15}, 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 (CoCl\textsubscript{2})-induced VEGF synthesis, possibly by diminishing HIF-1\textalpha synthesis in NPFs.

### METHODS

#### 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-Stammerberger modification for treatment of bilateral chronic sinusitis with sinonasal polyposis. Patients with a single polyp (antrochoanal polyp) or with other diseases related to nasal polyposis 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 (Optican 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.\textsuperscript{3,4,16} In brief, after removing the epithelial layer, the specimens were minced into 1-mm\textsuperscript{3} 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 100 \textsuperscript{5} cells/cm\textsuperscript{2}. Before different treatments, the cells were made quiescent by serum-free media (media without fetal calf serum) for 24 hours.\textsuperscript{3,4,16} Data given herein are the means of 8 experiments.

#### WESTERN BLOT ANALYSIS

Western blot analysis was performed as previously described.\textsuperscript{17} 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-1\textalpha (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 \beta-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, dehydrated, and subjected to antigen retrieved 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-1\textalpha (dilution 1:50; Novus Biologicals, Institute, Inc, Rockford, Illinois) and incubated with antibody–biotin conjugates (Vector Laboratories Inc, Burlingame, California) and 

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Biologicals, 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

CoCl2 STIMULATED VEGF SYNTHESIS IN NPFs

The amounts of VEGF produced in NPFs stimulated by CoCl2 (500 µmol/L) during various incubation periods were assessed by Western blot analysis. The results showed that CoCl2 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 CoCl2-STIMULATED HIF-1α

Cells were incubated with CoCl2 (500 µmol/L) for various incubation periods, and HIF-1α levels were determined by Western blot analysis. Western blot analysis revealed that the peak level of HIF-1α in NPFs treated with CoCl2 occurred at 4 hours and declined thereafter (Figure 2A and C). When NPFs were incubated with CoCl2, alone or in combination with EGCG (10 µg/mL) (3 hours before the addition of CoCl2) for 4 hours, HIF-1α levels were analyzed. Epigallocatechin-3-gallate significantly diminished CoCl2-induced HIF-1α synthesis (Figure 2B and D).

EGCG ABROGATED CoCl2-STIMULATED VEGF PRODUCTION BY REDUCING HIF-1α SYNTHESIS

To verify the involvement of HIF-1α in the synthesis of VEGF following CoCl2 stimulation, oligomycin, a specific inhibitor of HIF-1α, was applied in the subsequent experiment. 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. 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 is a well-established method of inducing changes similar to those seen with hypoxia.19,20 Immunohistochemistry of nasal polyp specimens confirmed the in vivo expression of VEGF in NPFs, in addition to the previously reported cellular sources of VEGF such as epithelial cells, endothelial cells, and infiltrating inflammatory cells.21 Furthermore, neovascularization adjacent to VEGF-positive NPFs was frequently found. Together, our results suggest that NPFs may contribute to the pathogenesis of nasal polyps by producing VEGF to promote angiogenesis in nasal polyps under hypoxic conditions.

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 significantly. A more profound inhibition was noted when EGCG and oligomycin were administrated together.

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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Author Contributions: Dr Liu had full access to all the data in the study and takes responsibility for the integ-

Figure 4. Immunolocalization of hypoxia-inducible factor-1α and vascular endothelial growth factor in nasal polyps. A, Hypoxia-inducible factor-1α was identified in fibroblasts (arrowheads) or mononuclear round cells (arrows) (Congo red, original magnification ×400). B, Vascular endothelial growth factor was clearly visible in fibroblasts, mononuclear round cells, and endothelial cells. C and D, Higher-powered magnifications of boxed areas of A and B, respectively. Note the intimate alignment between spindle-shaped vascular endothelial growth factor (brown granules)–producing fibroblasts (arrowheads) and the proliferating capillaries (arrows) (avidin-biotin-peroxidase complex, original magnification ×400).
rity of the data and the accuracy of the data analysis. Study concept and design: Liu. Acquisition of data: Lin, Shun, Kok, Wang, Hsiao, and Liu. Analysis and interpretation of data: Lin, Shun, Kok, Hsiao, and Liu. Drafting of the manuscript: Lin, Shun, Kok, Wang, Hsiao, and Liu. Critical revision of the manuscript for important intellectual content: Shun and Liu. Statistical analysis: Lin, Kok, and Hsiao. Obtained funding: Lin and Liu. Administrative, technical, and material support: Shun, Kok, Wang, Hsiao, and Liu. Study supervision: Shun and Liu.

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