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Gene expression profile of angiogenic factors in pulmonary arteries in COPD: relationship with vascular remodeling

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Submitted 30 July 2015; accepted in final form 15 January 2016

García-Lucio J, Argemi G, Tura-Ceide O, Diez M, Paul T, Bonjoch C, Coll-Bonfill N, Blanco I, Barberà JA, Musri MM, Peinado VI. Gene expression profile of angiogenic factors in pulmonary arteries in COPD: relationship with vascular remodeling. Am J Physiol Lung Cell Mol Physiol 310: L583–L592, 2016. First published January 22, 2016; doi:10.1152/ajplung.00261.2015.—Pulmonary vessel remodeling in chronic obstructive pulmonary disease (COPD) involves changes in smooth muscle cell proliferation, which are highly dependent on the coordinated interaction of angiogenic-related growth factors. The purpose of the study was to investigate, in isolated pulmonary arteries (PA) from patients with COPD, the gene expression of 46 genes known to be modulators of the angiogenic process and/or involved in smooth muscle cell proliferation and to relate it to vascular remodeling. PA segments were isolated from 29 patients and classified into tertiles, according to intimal thickness. After RNA extraction, the gene expression was assessed by RT-PCR using TaqMan low-density arrays. The univariate analysis only showed upregulation of angiopoietin-2 (ANGPT-2) in remodeled PA (P < 0.05). The immunohistochemical expression of ANGPT-2 correlated with intimal enlargement (r = 0.42, P < 0.05). However, a combination of 10 factors in a multivariate discriminant analysis model explained up to 96% of the classification of the arteries. A network analysis of 46 genes showed major decentralization. In this network, the metalloproteinase-2 (MMP-2) was shown to be the bridge between intimal enlargement and fibrogenic factors. In COPD patients, plasma levels of ANGPT-2 were higher in current smokers or those with pulmonary hypertension. We conclude that an imbalance in ANGPT-2, combined with related factors such as VEGF, β-catenin, and MMP-2, may partially explain the structural derangements of the arterial wall. MMP-2 may act as a bridge channeling actions from the main fibrogenic factors.

angiopoietin; pulmonary artery; COPD; vascular remodeling; gene expression profile

PULMONARY VASCULAR REMODELING is a frequent feature in smokers and patients with chronic obstructive pulmonary disease (COPD), mainly consisting of thickening of the intimal coat of pulmonary muscular arteries (3). Vascular remodeling is a dynamic process involving cellular and molecular changes, such as cell proliferation, cell migration, apoptosis, and changes in the synthesis or degradation of the extracellular matrix (21), which disturb both endothelial and smooth muscle cell homeostasis. These processes are highly dependent on the coordinated interaction of numerous growth factors and their corresponding receptors, which may be locally released or expressed in response to diverse stimuli, such as injury, hypoxia, or shear stress (11). In addition, there is evidence that bone marrow-derived circulating progenitor cells may be recruited at sites of vascular lesions and may take part in angiogenesis or tissue repair (26, 27). Progenitor cell fate is highly dependent on the microenvironment and, therefore, on the release of cytokines and growth factors. The signaling pathways of this phenomenon remain undetermined. Several data suggest that vascular remodeling may be the result of an angiogenic process often induced by chronic inflammation (4). Vascular endothelial growth factor (VEGF) is one of the key signaling molecules regulating the recruitment, proliferation, and migration of progenitor and/or endothelial cells and acts through specific receptors (19). Several other signaling systems are also involved in the regulation of the main steps during vessel formation and/or remodeling of preexisting vessels. For instance, matrix metalloproteinases (MMPs) influence angiogenesis by degrading matrix molecules and by activating or releasing growth factors sequestered within the extracellular matrix, such as VEGF, basic fibroblast growth factor (FGF), and insulin growth factor-1 (29). For its part, the formation of the vascular wall is an important step in vessel stabilization and maturation (21). Signaling platelet-derived growth factor system (PDGFB/PDGFβR), as well as angiopoietins (ANGPT-1, ANGPT-2, and their receptor TEK, also called Tie2) are involved in recruiting mural cells (pericytes and smooth muscle cells) (7, 37). The transforming growth factor (TGF)-β superfamily, which includes the bone morphogenetic proteins, has also been shown to be a critical regulator of pulmonary vascular remodeling in both secondary and idiopathic pulmonary hypertension (PH) (8, 28). Many of these factors may have effects on the proliferation and apoptosis of endothelial and/or smooth muscle cells. Taken together, the combination of factors/receptors involved in vascular remodeling remains a puzzling question.

Identification of the key molecules involved in the regulation of angiogenesis may provide new possibilities for the develop-
opment of suitable drugs for angiogenesis inhibition or stimulation in respiratory diseases, such as PH and emphysema. The aim of the present study was to examine the gene expression profile of 46 main growth factors, receptors, and key metalloproteinases in muscular pulmonary arteries obtained from lung resected specimens and to relate their expression with the degree of wall remodeling. A univariate analysis showed that remodeling of muscular pulmonary arteries was exclusively associated with higher expression of ANGPT-2. Nevertheless, a multivariate analysis showed that ANGPT-2 gene expression balanced with the expression of VEGF, MMP-2, and β-catenin (CTNNB-1) could partially explain the structural derangements of the arterial wall.

MATERIALS AND METHODS

Tissue Specimens

Surgical lung specimens were obtained from 29 subjects who underwent lobectomy or pneumonectomy for localization of a solitary pulmonary nodule. Subjects had normal lung function (n = 18) or criteria for COPD (n = 11), defined as a forced expiratory volume in 1 s (FEV1) < 80% and a FEV1-to-forced vital capacity ratio < 0.7. Patients with large-sized tumors were excluded from the study. The study was approved by our institution’s Committee on Human Research, and all subjects gave written, informed consent.

After lung resection, pulmonary arteries were selected from regions distal to the tumors to ensure that there was not an infiltration of cancer cells in the tissue sample. Pulmonary arteries adjacent to the distal to the tumors to ensure that there was not an infiltration of cancer cells in the tissue sample. Pulmonary arteries adjacent to the tumors were carefully dissected under microscope and cleaned of surrounding parenchyma and connective tissue. The histology of these pulmonary segments revealed the presence of an internal elastic lamina and an artery wall composed mainly of smooth muscle cells (Fig. 1). Two 3-mm-long rings were fixed in 4% paraformaldehyde, cryo-embedded in OCT, and frozen at −20°C.

Validation cohort. Additionally, plasma aliquots were obtained from a different set of subjects diagnosed with COPD (n = 62), with or without criteria for PH, and from healthy subjects (n = 48). Plasma was frozen and stored at −80°C until analysis.

Assessment of Vascular Remodeling

Morphometric characteristics of pulmonary muscular arteries were analyzed in formalin-fixed, paraffin-embedded lung tissue sections processed with elastic orcein stain. At least 10 arteries from each subject with complete elastic laminas were evaluated using a computerized image analysis system (Leica Qwin), as previously described (5). External and internal elastic laminas and the inner aspect of the intima were outlined. The areas occupied by the muscular layer, the intimal layer, and the lumen were expressed as percentages of the total area. A mean value of intimal thickening was obtained for each subject and used as a value of vascular remodeling.

Grouping Variables

According to the intimal thickening of pulmonary arteries, subjects were classified into tertiles: first tertile correspond to subjects with the “lowest degree of remodeling,” group R1; second tertile correspond to subjects with “medium degree of remodeling,” group R2; third tertile correspond to subjects with the “greatest degree of remodeling,” group R3.

RNA Extraction

Total RNA was extracted from arterial segments or lung tissues (~10 mg of tissue) using Trizol Reagent (Life Technologies, GIBCO BRL, Gaithersburg, MD), in accordance with the manufacturer’s instructions. RNA quality was checked with the LabChip Test kit using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

Gene Selection and Quantitative RT-PCR

Forty-six genes known to be modulators of the angiogenic process and/or involved in smooth muscle cell proliferation were studied. A list of the genes, grouped by families, is shown in Supplemental Table S1. (Supplemental material for this article is available online at the journal website.) Human β-actin and GAPDH were used as endogenous controls.

Specific TaqMan Gene Expression assays for each gene were selected from the manufacturer’s database (Applied BioSystems, Foster City, CA), and gene expression was determined by quantitative real-time PCR (qRT-PCR) with TaqMan low-density arrays (TLDA; Applied BioSystems).

TLDA

Each TLDA was configured with 48 genes by duplicate for four samples. Briefly, 2 ng of DNase-treated RNA from each sample were used for reverse transcription into 100 µl of cDNA. For each sample, 40 µl of synthesized cDNA were mixed with 210 µl of TaqMan universal PCR master mix (PE Applied Biosystems, New Jersey, NJ) and 170 µl of PCR water to form the reaction mix. Four hundred microliters of this mix were inserted on the microfluidic cards into mini-wells containing primers and probes of genes in duplicate. For each 384-well card, two matched cDNA samples were included at the same time for qRT-PCR reaction and analysis. qRT-PCR reaction and laser scanning were performed on an ABI 7900HT genotype with SDS2.1 software. Expression level of each gene was analyzed from the mean of its duplicates, and genes with reproducible amplification curves of both duplicates were analyzed and presented.

Array Validation by qRT-PCR

Selected genes underwent qRT-PCR for independent confirmation of relative expression levels in a Chromo 4 Real Time PCR detector (Bio-Rad, Hercules, CA) using the sensMix dt kit (Quantace, San Mateo, CA) based on the DNA double-strand-specific SYBR green I dye for detection. Results were normalized to the means of both GAPDH and β-actin expression levels, and relative gene expression was analyzed by the 2−ΔΔCt (cycle threshold) method. Primers were designed in accordance with human sequence database (Genome Browser).

Laser-Assisted Microdissection and Gene Amplification

Intrapulmonary arteries with diameters of 250–500 µm adjacent to the respiratory bronchioles were selected from lung tissue (n = 18) cryo-sections (16 µm thick) mounted on glass slides, and microdissected under optical control using a Laser Microbeam System (LEICA, Wetzlar, Germany). Briefly, after hemalaun staining for 45 s, the sections were immersed in 70% and 96% ethanol and stored in 100% ethanol until use. No more than 10 sections were prepared at once to reduce the storage time. Fifty arteries from each subject were identified and extracted. The external elastic lamina was outlined, and the arteries were then transferred into a reaction tube containing 65 µl of cold RNA lysis buffer.

Total RNA from microdissected arteries was purified using the RNeasy Micro Kit (Applied Biosystems), quantified with ND-2000 (Nanodrop), and qualified with 2100 bioanalyzer Agilent (xip picco). RNA was then reverse-transcribed using 0.25 µl of random primers in
20 μl of final volume reaction. Ten nanograms of cDNA were preamplified according to commercial guidelines using the TaqMan Gene Expression Assay Kit (Applied Biosystems) and the ANGPT-2 primer designed by TaqMan (Hs01048042_m1, Applied Biosystems). After 14 cycles of preamplification, the resulting product was diluted in Tris-EDTA buffer (1:20). PCR was performed with 2.5 μl of cDNA mixed with 0.5 μl of PCR Primers (20×) and 10 μl of TaqMan gene expression master mix. PCR was performed with 2.5 μl of cDNA in a GeneAmp 7900TH PCR cycler (Applied Biosystems).

**Immunohistochemistry**

Serial 2-μm-thick sections were cut from formalin-fixed OCT-embedded tissue blocks containing pulmonary artery rings. One slide from each series was stained with hematoxylin-eosin for light microscopic examination and artery location.

Sections were immunostained with different monoclonal antibodies using the avidin-biotin complex/horseradish peroxidase (HRP) method (K355; Dako, Glostrup, Denmark). Briefly, to inhibit peroxidase activity, sections were incubated with 0.5% hydrogen peroxide in methanol. After three washouts with phosphate-buffered saline (PBS), nonspecific binding was suppressed with normal goat serum followed by incubation of the following primary monoclonal antibodies: anti-human ANGPT-2 (R&D Systems), anti-smooth muscle α-actin (Dako), and anti-CD31 (Dako). After three washouts in PBS, sections were incubated with biotinylated rabbit anti-mouse immunoglobulin (E0354; Dako) followed by treatment with the avidin-biotin complex/HRP. Negative controls were done with nonimmune serum. Immunopositive cells were visualized by a solution of diaminobenzidine and hydrogen peroxide (Dako). The positive area and the intensity were evaluated by an analysis software (Image-Pro, Media Cybernetics, Buckinghamshire, UK) on 20 randomly selected fields of each subject (n = 29). The stained areas for each individual were normalized by the total area. An “Integrated Intensity Value” was calculated by multiplying the percentage of stained area by their intensity value in each image.

**Fig. 1.** Immunohistochemical staining of transverse sections of pulmonary arteries >1 mm obtained surgically from a patient with low remodeled arteries (R1; A, C, E, and G) and a patient with highly remodeled walls (R3; B, D, F, and H). A and B: pulmonary artery showing intense immunoreactivity to angiopoietin-2 (ANGPT-2). C and D: transverse sections stained with α-smooth muscle actin (SMA). E and F: immunohistochemistry against CD31 localized in the endothelium. G and H: negative controls.
ANGPT-2 Plasma Levels in COPD Patients

ANGPT-2 was assessed by sandwich ELISA (DuoSet, R&D Systems, Abingdon, UK) in plasma obtained from a cohort of patients diagnosed with COPD (n = 62; 17 current smokers and 45 former smokers) and compared with healthy controls (n = 48; 27 nonsmokers and 21 current smokers). Patients with COPD were subsequently divided into two subgroups: COPD with PH [COPD-PH (+); n = 16, 2 current smokers and 14 former smokers] and COPD without PH [COPD-PH (−); n = 46, 14 current smokers and 32 former smokers]. Briefly, R&D ELISA plates were incubated overnight with 100 μl of diluted capture antibody for ANGPT-2 in PBS (pH 7.2). This was followed by three washes with buffer (PBS, pH 7.2 and 0.05% Tween 20). Antigen blocking was done with 1% bovine serum albumin in PBS for 1 h. Recombinant proteins were serially diluted as recommended, to obtain a seven-point standard curve. All of the samples were diluted 1:2 in reagent diluent (R&D Systems). Samples and standards were incubated for 1 h at room temperature on a shaker at 500 rpm, followed by washing and addition of detection antibodies, which were incubated for 1 h. After four washes, streptavidin-HRP was added and incubated for 20 min. The substrate tetramethylbenzidine/H2O2 (Banthane, Genie, India) was added, and plates were incubated at room temperature in the dark for 20 min. Fifty microliters of stop solution (2N H2SO4) were then added to each well, and optical density was assessed at 450 nm.

Statistics

Data analysis was performed using Real-Time StatMiner software from Integromics (https://www.integromics.com). Relative quantification (RQ) was based on the comparative Ct method using GAPDH and β-actin as endogenous controls. For differential expression analysis between groups, a limma modified t-test (33) was used to calculate ΔΔCt quantities (ΔΔCt = mean ΔCt value (target samples) − mean ΔCt value (control samples)). RQ values were calculated from these ΔΔCt quantities (RQ = 2 − ΔΔCt) and used for fold-change computations. In parallel to this analysis, conventional statistical assessments of the data (RQ values) were also conducted for statistical tests by means of SigmaPlot (version 11.0; Systat Software, Chicago, IL) and SPSS (version 13.0; SPSS, Chicago, IL) packages.

Univariate analysis. Data are expressed as mean ± SD for normally distributed data or as medians and interquartile range for skewed distribution (Kolmogorov-Smirnov test). Statistical differences for each gene were assessed using the Kruskal-Wallis analysis of variance, and post hoc comparisons between groups were performed with the Dunn test.

Correlations. Correlations between variables were analyzed with Pearson’s coefficient. Probability values lower than 0.05 were considered as significant.

Multivariate analysis. DISCRIMINANT ANALYSIS. A linear discriminant function analysis with cross-validation was used to determine which variables best discriminate between groups. We assumed the degree of remodeling as the categorical variable, obtained by classifying pulmonary arteries into three groups according to the intimal thickness using the tertiles as cutoff values. The performance of the analysis was evaluated by leave-one-out cross-validations. Post hoc, the relative strength of selected variables in this analysis was checked by multiple linear regression analysis.

Multiple linear regression. We performed a regression analysis using as explanatory variables the –Ct values of genes resulting from the previous discriminant analysis variables and the percentage of intima as the independent variable. In this analysis, each gene was previously evaluated for outliers and removed before running the test.

After the multiple regression analysis of genes selected from discriminant analysis, the explanatory variables (genes) used were evaluated for multicollinearity using the variance inflation factor and removed from the equation if they were above 4. Lastly, no significant variables (P > 0.05) on the model were removed from the equation.

Network analysis. A network was constructed using the gene expression matrix correlation obtained after a Pearson analysis between each pair of genes. The network generated was compiled in simple interaction format amenable to Cytoscape for further topological analysis (32). In this network, nodes represent the genes, and the network edges (connecting lines) are depicted when P denotes a significant correlation (P < 0.05) with each other. The size of nodes is proportional to the betweenness centrality, whereas the thickness of edges is constructed from the edge betweenness value between each pair of genes. The betweenness centrality of a node reflects the amount of control that this node exerts over the interactions of other nodes in the network, while edge centrality represents a bridge-like connector between two parts of a network, the removal of which may affect the communication between many pairs of nodes through the shortest paths between them.

RESULTS

Subjects

Eleven patients with COPD and 18 subjects with normal lung function (9 nonsmokers and 9 smokers) were enrolled in the study (Table 1). Patients with COPD had significantly lower FEV1, forced vital capacity-to-FEV1 ratio, and diffusing capacity for carbon monoxide than healthy controls. Groups formed according to their degree of remodeling were matched for subjects with COPD and subjects with normal lung function. There were no differences in respiratory variables between groups.

The histology of these pulmonary segments revealed the presence of an internal elastic lamina and an artery wall composed mainly of smooth muscle cells (Fig. 1).

After RNA extraction, we studied the expression profile of 46 genes, including growth factors, their receptors, MMPs, and cytokines in 29 subjects, according to the degree of remodeling by means of TLDAs, which were run in parallel to the 48 qPCR. After normalization of gene expression, we performed several bioinformatics approaches to analyze the data in relation to the degree of pulmonary vascular remodeling by grouping the subjects into tertiles, according to their intimal thickness.

Univariate Analysis

Table 2 shows the fold change in all genes studied according to their degree of remodeling. Results of the TLDAs showed that ANGPT-2 gene expression increased significantly in both group R2 and R3 pulmonary arteries compared with the ones

| Table 1. General characteristics of the population (nonsmokers, smokers, and COPD) |
|-----------------|-----------------|-----------------|
| Nonsmokers | Smokers | COPD |
| n | 9 | 9 | 11 |
| Age, yr | 66 ± 7 | 64 ± 8 | 62 ± 5 |
| Tobacco, packs/yr | 0 | 63 ± 32† | 66 ± 32† |
| Smoking status (current/former) | N/A | 6/3 | 8/3 |
| FEV1, %predicted | 100 ± 15 | 92 ± 11 | 66 ± 12‡ |
| FEV1/FVC, % | 74 ± 5 | 77 ± 5 | 56 ± 8† |
| DLCO, %predicted | 92 ± 14 | 77 ± 18 | 74 ± 2‡ |
| PaO2, mmHg | 88 ± 15 | 88 ± 9 | 75 ± 9 |
| Intimal thickness, %total area | 19 ± 6 | 24 ± 7 | 23 ± 9 |
| R1/R2/R3 distribution | 4/2/2 | 2/4/3 | 3/4/4 |

Values are means ± SD; n, no. of subjects. COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; DLCO, diffusing capacity for carbon monoxide; PaO2, arterial O2 partial pressure; R1, R2, and R3: subjects with the lowest, medium, and greatest degree of remodeling, respectively; N/A, nonapplicable. †P < 0.05 vs. nonsmokers. ‡P < 0.05 vs. smokers.
with low remodeling (group R1). TEK gene expression was lower and MMP-2 gene expression was higher in remodeled arteries, although the differences were not statistically significant (Table 2). Immunohistochemistry results also showed that in both R3 and R2, ANGPT-2 expression was significantly higher in cells lying in the intima and within the endothelium of pulmonary artery rings (Fig. 1) than in the R1 group (integrated intensity value for R3: 3,593 ± 940; R2: 3,291 ± 1,617; R1: 1,713 ± 887; ANOVA, P < 0.01).

Correlations

Protein expression of ANGPT-2 assessed by immunohistochemistry correlated directly with the degree of remodeling assessed by planimetry (Fig. 2A). Moreover, gene expression of ANGPT-2 in arteries of middle size (1- to 2-mm diameter) obtained surgically correlated directly with those narrower arteries (0.2- to 0.5-mm diameter) obtained by laser microdissection, thus reflecting a concordance in two different size segments of the pulmonary vascular tree (Fig. 2, B and C). Gene expression of ANGPT-2, in both middle- and small-sized arteries, also correlated with the intimal enlargement (r = 0.54 and r = 0.49, respectively; P < 0.05).

Multivariate Analysis

**Discriminant analysis.** We used discriminant analysis to study the specific distribution pattern of 46 growth factors and receptors, according to the degree of remodeling of the pulmonary arteries. The analysis revealed three distinct clusters of growth factor distribution: less remodeled (R1), medium remodeled (R2), and highly remodeled (R3) pulmonary arteries (Fig. 3). Figure 3A shows the coefficients of 10 genes in the two canonical functions generated by the analysis: ANGPT-2,
**CASP3** (caspase-3), **CTNNB-1**, **EDNRB** (endothelin receptor B), **MMP-2**, **PDGFRB** (platelet-derived growth factor receptor B), **TEK**, **TGFBR-1** (transforming growth factor B receptor-1), **TIMP4** (tissue inhibitor of metalloproteinase-4), and **VEGF** (vascular endothelial growth factor). The values for discriminant functions 1 and 2 (D1 in X-axis; D2 in Y-axis) for each individual were obtained as a linear function of 10 variables [**ANGPT-2**, **CASP3** (caspase-3), **CTNNB-1** (β-catenin), **EDNRB** (endothelin receptor B), **MMP-2** (metalloproteinase-2), **PDGFRB** (platelet-derived growth factor receptor B), **TEK**, **TGFBR-1** (transforming growth factor B receptor-1), **TIMP4** (tissue inhibitor of metalloproteinase-4), and **VEGF** (vascular endothelial growth factor)], where \( D = u_1X_1 + u_2X_2 + \ldots + u_{10}X_{10}. \)

The coordinates of centroid's group are calculated, resolving the equations with the mean values of each variable in each group. **B**: table with leave-one-out, cross-validation values. R2, group with medium degree of remodeling.

**Network Analysis**

To better understand the underlying mechanism of intimal thickening, a gene network was built using the matrix correlation of gene expression for each pair of genes. This network inference and its simple parameters are shown in Fig. 4. The network constructed from 46 genes showed substantial decentralization. However, the notable edge betweeness (proportional to the edge thickness) connecting the intimal enlarge-
ment and MMP-2 indicates the influence of the latter on vascular remodeling.

**Determination of ANGPT-2 Plasma Levels**

Analysis of ANGPT-2 plasma levels in another cohort of patients with COPD revealed higher values in this group than in nonsmokers (Table 4). In the COPD group, current smokers showed increased levels of ANGPT-2 than former smokers (Fig. 5A). Interestingly, in a subsequent analysis, COPD patients with PH showed higher values of ANGPT-2 ($P < 0.05$) than those without (Fig. 5, B and C). Moreover, in COPD patients without PH, plasma levels of current smokers were higher than those of former smokers (Fig. 5C). In addition, we found a significant correlation of ANGPT-2 plasma levels and the number of packs/year (Fig. 5D).

**DISCUSSION**

In this study, we evaluated the gene expression profile of a selected family of growth factors and receptors in muscular pulmonary artery segments obtained from lung resected specimens. We related their expression with the degree of vessel wall remodeling.

The univariate analysis revealed few changes in the gene expression pattern of growth factors in remodeled and nonremodeled arteries. The most significant change observed was in ANGPT-2, whose level of expression increased almost threefold in the medium and highly remodelled arteries. The precise role of increased ANGPT-2 in the remodeled arteries in these subjects is unknown. Usually, this protein is considered as an antagonist of the angiogenic factor, ANGPT-1, acting as a stabilizer of the endothelium (11, 34). Therefore, the effects observed in remodeled vessels may be due to a deficit of ANGPT-1 rather than a direct effect of ANGPT-2. The in vivo effects of elevated ANGPT-2 have been studied in animal models (10, 24). In these studies, when ANGPT-2 was overexpressed, a reversion of vasculature was observed toward a more plastic state, providing stabilization signals to promote sprouting and remodeling (24, 35). These signals may loosen the vessel structures, reducing endothelial cell contacts with the extracellular matrix (31) and disassociating the peri-endothelial support cells. Interestingly, these findings are in accordance with the instability of the endothelium and proliferating intimas observed in PA of subjects with COPD (27, 30), as well as in advanced atherosclerotic lesions in coronary arteries (9). However, in addition to the inhibition of ANGPT-1-dependent responses in endothelial cells, ANGPT-2 may act as a real agonist in smooth muscle cells mediated by the same TEK receptor. Pulmonary artery smooth muscle cell treated in vitro with ANGPT-2 showed an increase in the expression of $\alpha$-actin and greater survival of these cells in accordance with a greater muscularization of pulmonary arteries (data not shown). Also, Yuan et al. (38) demonstrated in vitro that ANGPT-2 is able to induce phosphorylation of TEK, causing downstream effects in endothelial cell, although these effects may be weaker than those produced by ANGPT-1.

The multivariate analysis demonstrated that a combination of 10 factors explained up to 96% of the classification of arteries, according to the degree of vascular remodeling. Of these 10 factors, five showed a multiple correlation between them, ANGPT-2, MMP-2, VEGF, TEK, and CTNNB-1 explained 52.0% of the variability of the intimal thickness, and $\beta$-catenin was the factor with the most weight in the equation. Although individually these factors have been related in some way with vascular remodeling, the combination of several factors may be of greater physiological relevance for revealing potential synergistic actions. For instance, it has been suggested that the loss of vessel structures might make endothelial cells more accessible and responsive toward the angiogenic inducer VEGF (and probably to other inducers as well) (18). It is possible then that endothelial cells in the presence of VEGF (or another angiogenesis inducer) may become activated to migrate and proliferate, producing new capillary sprouts and tubule structures. The continuous presence of ANGPT-1 could allow a shift in the local balance of ANGPT-1/ANGPT-2 back in favor of ANGPT-1, affecting the maturation and stabilization of the newly formed vessels. There appears to be a collaboration between VEGF, ANGPT-2, and ANGPT-1 to elicit angiogenesis (35). In contrast, vascular regression is associated with higher levels of ANGPT-2 expression in the absence of the activating (survival) signal from VEGF, which presumably overwhelm the ANGPT-1 signal and thus produce a catastrophic detachment from the matrix and supporting cells, likely resulting in cellular apoptosis. This assumption has been tested in experimental studies of endothelial cell cultures and explanted veins (31) in which exogenous ANGPT-2 caused endothelial cell detachment. Interestingly, in our study, the multiple linear regression analysis revealed an interrelationship between ANGPT-2, VEGF, and TEK, which was suggestive of this scenario. In this model, the increase in ANGPT-2 expression seems to run in parallel to the reduction of TEK and VEGF expression, a situation that suggests inhibition of ANGPT-1. On the other hand, little is known about the potential synergism of angiopoietin and MMP-2. It is recognized that MMPs play a role in the regulation of cyto-

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**Table 3. Multiple regression analysis of genes selected from discriminant analysis**

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Standard Error</th>
<th>t</th>
<th>$P$</th>
<th>Variance Inflation Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>47.269</td>
<td>9.525</td>
<td>4.963</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ANGPT2</td>
<td>2.390</td>
<td>0.820</td>
<td>−2.792</td>
<td>0.010</td>
</tr>
<tr>
<td>MMP2</td>
<td>2.607</td>
<td>1.117</td>
<td>−2.334</td>
<td>0.028</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>18.799</td>
<td>5.263</td>
<td>−3.572</td>
<td>0.002</td>
</tr>
<tr>
<td>TEK</td>
<td>−6.342</td>
<td>2.187</td>
<td>2.900</td>
<td>0.008</td>
</tr>
<tr>
<td>VEGFA</td>
<td>−3.984</td>
<td>1.452</td>
<td>2.744</td>
<td>0.011</td>
</tr>
</tbody>
</table>

All independent variables appear to contribute to predicting percent intimal thickness (% INT), as % total area ($P < 0.05$).
kines, chemokines, and growth factors (4, 17). MMP-2 has been implicated in tissue remodeling, and the present results suggest a positive relationship between these two proteins. The expression of MMP-2 showed a positive correlation with the enlargement of the intima, stressing the importance of this association in vascular remodeling. Network analysis also revealed that the expression of MMP-2 was directly linked to the expression of main fibrogenic growth factors, such as FGF, hepatocyte growth factor, and bone morphogenetic protein-2, indicating that this node represents an important bridge in the signaling cascade during vascular remodeling.

VEGF-mediated disruption of cell-cell interactions has been attributed to the dissociation of β-catenin from VE-cadherin (36). Interestingly, this effect may be opposed by ANGPT-1, as it specifically counteracts the ability of VEGF to induce the phosphorylation-dependent redistribution of VE-cadherin, thereby rescuing the endothelial barrier function (15). In fact, the expression of β-catenin was related directly to the expression of TEK receptor. The potential involvement of the Wnt/β-catenin pathway has also been highlighted in atherosclerosis (13). Interestingly, the analysis of human carotid artery atherosclerotic plaques revealed high levels of cell proliferation and active β-catenin in disrupted plaques compared with stable plaques, suggesting a role for β-catenin signaling in smooth muscle cell proliferation within atherosclerotic plaques (6). In our study, β-catenin is an important node in the signaling network connecting with multiple growth factors regulating cell proliferation, with FGF2 presenting the strongest associ-

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**Table 4. Plasma ANGPT-2 levels in COPD**

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>COPD</th>
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<tbody>
<tr>
<td><strong>n</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>57 ± 8</td>
<td>55 ± 7</td>
<td>60 ± 6†</td>
</tr>
<tr>
<td>Tobacco, packs/yr</td>
<td>3 ± 2</td>
<td>31 ± 24*</td>
<td>64 ± 28†</td>
</tr>
<tr>
<td>Smoking status (current/former)</td>
<td>0/8†</td>
<td>21/0</td>
<td>17/45</td>
</tr>
<tr>
<td>FEV₁, %predicted</td>
<td>106 ± 14</td>
<td>101 ± 12</td>
<td>45 ± 21†</td>
</tr>
<tr>
<td>FEV₁/FVC, %</td>
<td>78 ± 5</td>
<td>77 ± 5</td>
<td>41 ± 14†</td>
</tr>
<tr>
<td>DLCO, %predicted</td>
<td>91 ± 15</td>
<td>84 ± 9</td>
<td>53 ± 19†</td>
</tr>
<tr>
<td>ANGPT-2, ng/ml</td>
<td>804 ± 362</td>
<td>1,036 ± 533</td>
<td>1,174 ± 706*</td>
</tr>
</tbody>
</table>

Values are means ± SD. n, no. of subjects. †10 Nonsmokers have never smoked. *P < 0.05 vs. nonsmokers. †P < 0.05 vs. smokers.
FGF2 has also been associated with intimal hyperplasia in atherosclerosis (20).

The majority of arteries used in this study were obtained from heavy smokers, and previous studies have associated cigarette smoke exposure with greater remodeling in COPD (30). Also, several studies have shown that COPD is associated with oxidative stress, activation of circulating inflammatory cells, and increased levels of proinflammatory cytokines (1, 2, 5). Interestingly, ANGPT-2 expression was upregulated in skeletal muscles of COPD patients, likely due to oxidative stress (25). In accordance with this, plasma levels of ANGPT-2 in COPD former smokers vs. current smokers showed increased values in the latter, also suggestive of a potential effect of oxidative.

Several studies have related circulating angiopoietins to the outcome and severity of various illnesses and have suggested that they may be prognostic biomarkers for survival. In patients with sepsis and septic shock, ANGPT-2 levels in plasma increased and correlated with disease severity, whereas ANGPT-1 levels fell (16). ANGPT-2 concentrations increase in acute coronary syndromes (12), congestive heart failure (22), and cardiogenic shock (23). Since ANGPT-2 was increased in remodeled pulmonary arteries, we hypothesized that ANGPT-2 could be related to PH in COPD, because this condition is associated with the enlargement of the pulmonary arterial wall. We, therefore, analyzed the plasma levels of ANGPT-2 in a cohort of COPD patients previously diagnosed with PH by echocardiography. The analysis showed a significantly higher level of this cytokine in patients diagnosed with PH. Taking into account our results, the increased level of this protein may be related to a higher degree of pulmonary vascular remodeling, which might recognize ANGPT-2 as a novel biomarker. Interestingly, the increase in ANGPT-2 in PH associated with COPD contrasts with the high levels of ANGPT-1 found in nonfamilial pulmonary artery hypertension and suggests that different mechanisms occur in each condition (14). This finding indicates the need for longitudinal studies evaluating circulating levels of ANGPT-2 before and after treatment of COPD.

In summary, our findings show that pulmonary vascular remodeling is associated with increased expression of ANGPT-2. Higher plasma levels of ANGPT-2 were associated with PH in COPD and with cigarette smoke. An imbalance of ANGPT-2 with related factors, such as VEGF, CTNNB-1, and MMP-2, may partially explain the structural derangements of the arterial wall. MMP-2 may act as a bridge, channeling actions from the main fibrogenic factors.

ACKNOWLEDGMENTS

We thank Jose Ramirez, Susana Maqueda, and Núria Aventin for expert technical assistance, and Raquel Bermudo from the technical staff of Laser Dissection Laboratories for skillful support during the study. The authors also thank Michael Maudsley for the English revision of the manuscript. This work was developed at the Centre de Recerca Biomèdica Cellex, Barcelona, Spain.
REFERENCES


