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The effects of hypoxia on the expression of vascular endothelial growth factor in broiler lung fibroblasts

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Abstract: The aim of this study was to investigate the effects of hypoxic stresses on vascular endothelial growth factor (VEGF) in cultured lung fibroblasts of meat-type chickens. Isolated and subcultured primary lung fibroblasts were exposed to hypoxia. The presence of VEGF and the effects of hypoxia on cultured lung fibroblasts and in the culture media were evaluated. Immunocytochemistry using streptavidin-biotin-peroxidase method and ELISA using commercially available primary monoclonal antibodies were used for evaluation in both 6-h and 12-h hypoxic fibroblasts. The level of VEGF was slightly higher in the 6-h hypoxic group (1.22 ng/mL) than in the 6-h control group (1.27 ng/mL) in cell culture media by ELISA. In the 6-h hypoxic group, a correlation between the decrease in immunocytochemical score and increase in medium with VEGF was observed. The presence of VEGF was shown in cultured chicken lung fibroblasts from meat-type chickens. In conclusion, a significant decrease in immunocytochemical staining score was observed along with a slight increase in the amount of VEGF in culture media under hypoxic conditions. The results suggest that VEGF might have a role in edema formation via permeability increase in the lungs of ascitic meat-type chickens.

Key words: Vascular endothelial growth factor, ascites, meat-type chicken, hypoxia

1. Introduction

Ascites (pulmonary hypertension), is a metabolic disorder that causes economic losses and is characterized by the accumulation of lymph fluid in the abdomen due to right heart failure in meat-type chickens. At necropsy, besides the lesions observed, other common findings such as generalized edema and expansion of the air capillaries are typically observed in the lung tissues of the chickens (1). Although environmental factors such as cold, high altitude, and diet play significant roles in the development of the syndrome (2), the main cause is hypoxia caused by increased oxygen demand, pulmonary hypertension, and extra workload on the heart (1–4).

Vascular endothelial growth factor (VEGF), which is also known as edema factor, is a potent and key factor in the development of tumoral angiogenesis and edema formation by increasing vascular permeability (5,6). There are five VEGF products of alternative splicing, VEGF 121, VEGF 145, VEGF 165, VEGF 189, and VEGF 206 (7). VEGF 121 and VEGF 165 are secreted and both have mitogenic and permeability-inducing properties. VEGF has been synthesized in both animals and humans in endothelial cells, in mesenchymal cells, in alveolar macrophages of lung, in neurons, in renal epithelial cells, in connective tissue cells, and in tumor cells (8). Hypoxia has an important role in triggering VEGF (5,9). Investigations on people living at high altitudes showed that there might be a direct relationship between hypoxia and VEGF level due to the observed higher levels of serum VEGF, while in vitro studies on VEGF revealed that fibroblasts inflicted with mechanical damage and exposed to hypoxia showed an increase in VEGF levels compared with the control group (9).

During the formation of pulmonary edema, matrix metalloproteinases (MMPs) and their relation with hypoxic stress were examined through both in vivo and in vitro studies for pulmonary hypertension syndrome (10,11). It was shown that MMPs have the potential for damaging the vascular basement membrane, but the mechanisms by which hypoxia causes ascites by increasing vascular permeability remains unclear. Thus, the aim of this study is to investigate the effects of hypoxic stress on VEGF, also known as edema factor, and its contribution to the pathogenesis of the disease in cultured lung fibroblasts of meat-type chickens.

2. Materials and methods

2.1. Cell culture

This study was carried out after receiving written approval from the Scientific and Ethics Committee of Uludağ

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University. Three male broiler chicks (Ross 308) of 1 day old were obtained from a local commercial hatchery (HasTavuk A.Ş., Bursa, Turkey). After transportation to the laboratory, chicks were accommodated for approximately 1 h and then were euthanized by ether inhalation. The body of the birds was washed with 70% alcohol solution for the elimination of environmental contaminants. After the removal of abdominal feathers from the body, skin was rinsed with 70% alcohol again and incised from the thoracic inlet to cloaca. Skin samples, 1 cm × 1 cm in size, from the thoracal region were collected into 15-mL centrifuge tubes containing Krebs ringer bicarbonate buffer solution (transfer solution) (K4002, Sigma, St. Louis, MO, USA) with 5× antibiotic-antimycotic solution (P3539, Sigma). Regional muscles were dissected and the body cavity was opened while preventing any contamination. Lung fibroblast cells were isolated using a modification of the procedures described by Hewett and Murray (12) and by Twal and Leach (13). Briefly, lungs were dissected out and transferred into a 15-mL centrifuge tube containing transfer solution. The collected skin and lung samples were rinsed three times in transfer solution. Pleura were dissected out to eliminate possible contamination by mesothelial cells. Samples were then chopped carefully with two scalpel blades to cubes of about 2 mm under a class II biological safety cabinet (Jouan, France). All 6-well plates and tissue culture flasks were coated with 2% gelatin (G9391, Sigma) to improve attachment. Two or three pieces of explants were placed onto each other in wells of the gelatin-coated 6-well plates (Greiner, Germany) and incubated for 10 min at 37 °C to allow them to attach. One milliliter of complete culture medium (M199) (M3769, Sigma) with 20% fetal bovine serum (FBS; F4135, Sigma) and 2% antibiotic-antimycotic (P3559, Sigma) was added over the explants and wells were incubated at 37 °C in a humidified atmosphere in a 5% CO_2 cabinet under normoxic conditions (Jouan). The medium was changed every other day.

Lung tissues from three birds were used for enzymatic disaggregation. After mincing as described above, tissue fragments were put into a 15-mL centrifuge tube containing 3 mL of collagenase solution (0.036 g of collagenase (C6885, Sigma), 1.35 g of bovine serum albumin (A9418, Sigma) and 0.038 g of dispase (17105 - 032, GIBCO, Paisley, UK)) in 18 mL of Krebs ringer bicarbonate solution. Tissue homogenate was incubated with the enzyme solution for 2 h at room temperature on a shaker. After incubation, 3 mL of a heparin (H3149, Sigma) solution (0.0575 g of heparin in 20 mL of M199 medium) was added to each tube. The digests were then filtered through ethylene oxide gas-sterilized 100-µm nylon mesh and collagenase activity was stopped by the addition of 3 mL of M199 medium containing 10% FBS. The filtrate was centrifuged at 1000 rpm for 10 min at room temperature. The pellets were resuspended in 5 mL of complete medium and plated into gelatin-coated 25-cm^2 tissue culture flasks (Costar - Corning, Tewksbury, MA, USA) and incubated at 37 °C in a humidified atmosphere of a 5% CO_2 cabinet. Medium was changed every other day.

The cells were subcultured when they completely covered the flask and began to pile up (confluency). Briefly, medium was removed and culture plates were washed with 2 mL of preheated 37 °C Krebs ringer bicarbonate buffer solution for 2 min. The washing buffer was then removed and 2 mL of preheated 0.04% trypsin solution was added. Culture plates were incubated in the CO_2 incubator for 10 min at 37 °C. At the end of the incubation, trypsin activation was stopped with the addition of an equal volume of complete culture medium. After the trypsinization, cells from each well of the 6-well plate were seeded in 25-cm^2 flasks and incubated under the same conditions mentioned above.

2.2. Characterization of cells

Before the second passage, the cells were seeded onto 1.5-cm-diameter cover slips placed into 24-well plates and incubated in a humidified atmosphere of 5% CO_2. After attachment, cells were grown to approximately 80% confluency and characterized immunohistochemically using monoclonal antibodies against vimentin (Dako, Glostrup, Denmark) and cytokeratin (Novocastra, Newcastle upon Tyne, UK). Skin fibroblasts of broiler chickens from explant cultures were used as a positive control.

2.3. Hypoxic treatment

Hypoxic conditions were generated using the AnaeroGen system (Oxoid, Basingstoke, UK) (14). Briefly, chicken lung fibroblast cells (3×10^4 cells) were seeded on 1.5-cm-diameter cover slips placed in the wells of 24-well plates and incubated at 37 °C for 24 h. When they reached confluency, they were placed in a 2.5-L air-tight jar (Oxoid) with an AnaeroGen sachet (AN25, Oxoid). The lid of the jar was closed immediately and the jar was incubated at 37 °C in the humidified atmosphere of a 5% CO_2 cabinet until opening. The experiment groups were designated as control, 6-h hypoxia treatment, and 12-h hypoxia treatment groups.

2.4. Collection of medium samples

At the end of each hypoxic treatment period, the culture medium of each well was collected aseptically in a class II biological safety cabinet into sterile Eppendorf tubes and used for ELISA.

2.5. Immunohistochemical staining

The cells (3×10^4/well) were seeded onto round cover slips placed into the wells of 24-well plates and incubated at 37 °C in a humidified atmosphere of a 5% CO_2 cabinet. The

cells, when they reached 80% confluency, were stained with commercially available mouse antihuman VEGF (RB - 9031, P1, Thermo Scientific, Fremont, CA, USA) using a modified streptavidin-biotin-peroxidase complex (Strep-ABC) method. Briefly, after the removal of medium, the cells were washed with PBS (pH 7.4) for 5 min. The PBS was then decanted and cells were fixed with 0.5 mL of cold acetone (–20 °C) for 5 min. Plates were incubated for approximately 3 min at ambient temperature for the evaporation of excess acetone and rinsed with PBS for 2 × 5 min. Protein blocking solution was applied for 10 min. Primary antibody, diluted 1:100 in PBS, was applied for 1 h. The cells were then rinsed in PBS and biotinylated secondary goat antimouse antibody (LabVision, Fremont, CA, USA) was applied for 30 min. After incubation with streptavidin-peroxidase (LabVision) for 30 min, aminoethyl carbazole and diaminobenzidine were applied for 15 min as chromogen. The slides were finally counterstained with Mayer's hematoxylin or Harris hematoxylin and mounted with glycerin gel or Entellan. Hemangiosarcoma was used as a positive control. Negative controls were achieved by replacing the primary antibody with PBS. The same procedure was also followed for the characterization of isolated lung fibroblasts, which were stained with cytokeratin and vimentin.

The number of stained cells and intensity of the staining, in eight randomly selected areas of at least 400 cells each at 400× magnification, were evaluated for scoring from 0 to 4 + in a semiquantitative system (11, 12). In brief, the scores and their expression were 0: unstained, + : faint, + + : moderate, + + + : strong, or + + + + : very strong. Staining results were averaged and presented as bar graphs. All slides were evaluated in a blinded manner by two of the authors (AA and MÖÖ) separately and, in the case of disagreement, the slide was reevaluated.

2.6. ELISA

The media samples were stored at –20 °C until assayed. Each was dissolved and brought to laboratory temperature. The standard curve concentrations took place between 0.625 and 10 ng/mL. Standards and samples from each of experimental group were placed in the well and incubated at 37 °C for 1 h. Wells were washed with an automated washer (Thermo Scientific, Wellwash, 4MK2) five times, and then 50 µL of HRP-Avidin was added to each well and wells were incubated at 37 °C for 30 min. After washing, 50 µL of Substrate A and B were added and wells were exposed in a dark environment for 15 min. Next, 50 µL of stop solution was added to each well and the optical density of each well was determined within 10 min using a microplate reader (Thermo Scientific, Multiscan FC) set to 450 nm.

2.7. Statistical analysis

Mean values for VEGF-immunocytochemistry activity and ELISA results were calculated from experiments in each cell line and for each time point. The data were analyzed by Wilcoxon's rank sum and Mann–Whitney U tests using SPSS version 13.0 (SPSS Inc., Chicago, IL USA). $P < 0.05$ was considered significant.

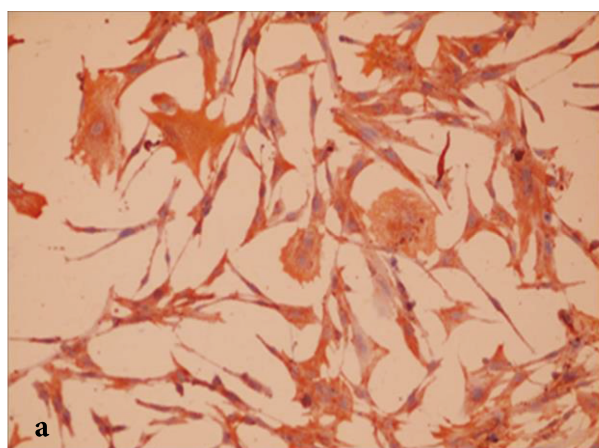
3. Results

3.1. Characterization of cells

Fibroblast cells isolated from the lung and skin tissue were stained positive for cytokeratin and negative for vimentin (Figures 1a and 1b). Morphologically confluent cells were spindle-shaped cells confirming their identity as fibroblasts (Figures 2a and 2b).

3.2. Staining with VEGF antibody

Primary fibroblasts isolated from lungs of meat-type chicken were stained intracytoplasmically with VEGF antibody (Figures 3a–3c).



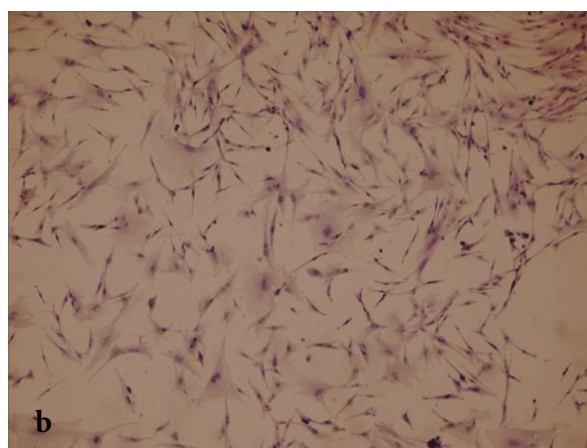


Figure 1a. Lung fibroblasts, positively stained with vimentin, 20× original magnification. **b.** Lung fibroblasts, negatively stained with cytokeratin, 4× original magnification.

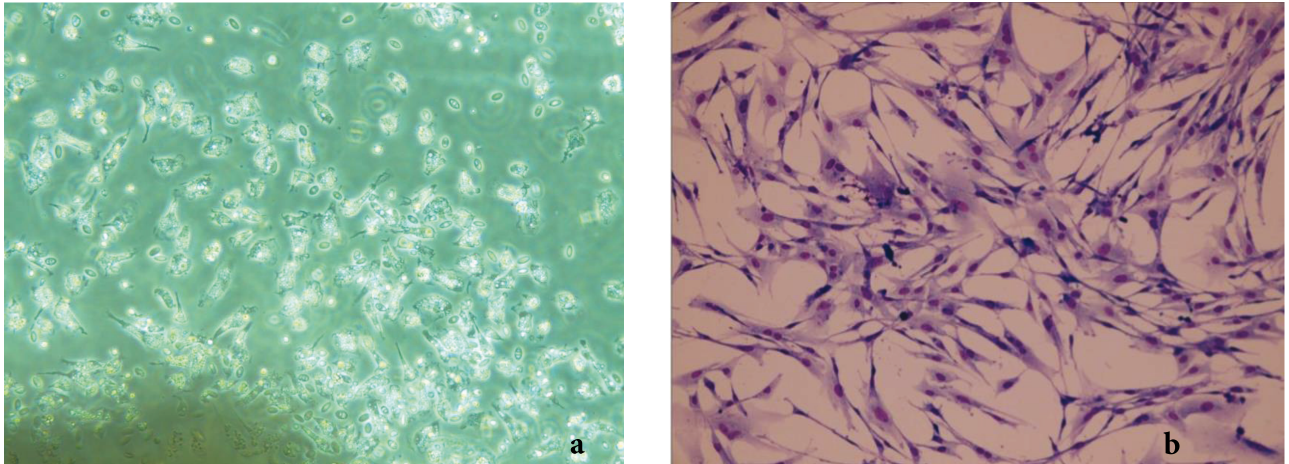


Figure 2a. Phase-contrast micrograph of confluent lung fibroblasts, 20× original magnification. **b.** Lung fibroblasts, stained with hemacolor, 20× original magnification.

Figure 3a. VEGF staining in the cytoplasm of lung fibroblasts cells. Strep-ABC, 0-h control fibroblast cells, 40× original magnification. **b.** Decreased VEGF staining in the cytoplasm of lung fibroblasts cells. Strep-ABC, 6-h hypoxic fibroblast cells, 40× original magnification. **c.** Mild increased VEGF staining in the cytoplasm of lung fibroblasts cells. Strep-ABC, 12-h hypoxic fibroblast cells, 40× original magnification

3.3. Immunocytochemical scoring results

The mean staining scores of fibroblasts are shown in Table 1. When compared to the start time (0 h), 6 and 12 h of mean staining scores in the control group showed a decrease at the end of 6 h. Otherwise, at the end of 12 h, the mean staining scores had increased to the level of the start time. When hypoxic groups were compared to each other, there was a low mean staining score at the end of 6 h compared with the start and the 12-h group ($P < 0.01$). Compared with their own control groups, for fibroblasts exposed to both 6-h and 12-h hypoxic periods, the mean staining scores were lower than the starting point mean staining scores ($P < 0.01$).

3.4. ELISA results

Measurements of medium at 450 nm from fibroblasts subjected to hypoxia are shown in Table 2 as ng/mL. In both control and hypoxic groups, while starting and 12-h group values were close to each other, there was an increase in measurements for the 6-h hypoxic group. In both groups there was a slight increase compared to the control, although these differences did not quite reach statistical significance ($P < 0.5$).

4. Discussion

VEGF is an important autocrine growth factor contributing to development of the circulatory system (vasculogenesis) and the formation of blood vessels (angiogenesis) (5,15) and improvement of the pulmonary capillary beds and the blood/air barrier in the lungs (16). Physiologically, immunohistochemical staining of pulmonary parenchymal cells with VEGF has been shown as essential to keep a balance between microvascular permeability and endothelial cells (6). However, in some pathological conditions like hypoxia, VEGF is known to play important roles in alveolar and bronchial vessels (8,17). It was shown that during high-altitude hypoxic stress, VEGF expression is upregulated (18,19). Acute exposure of hypoxia leads to an increase in lung water content and total protein and albumin leakage in lavage fluids. The increased water content in lungs exposed to hypoxia appears to be due to leakage of fluids from intravascular to extravascular fluid compartments (8,20,21). Shukla et al. (21) showed that bronchoalveolar fluid in lungs exposed to hypoxia for 5 h had a 1.5-fold increased VEGF level compared to the control group. Many in vitro studies showed a clear link between hypoxia-induced VEGF and angiogenesis (22). It was proven that healthy and injured fibroblasts express and secrete VEGF under pathological conditions (23). In addition, fibroblasts may play an important role in the vascular response to hypoxic injury and have the ability to rapidly respond to hypoxic stress (20). Fibroblasts from fetal lungs or mature mammary tissues exposed to hypoxia showed a 4- or 5-fold increase in VEGF secretion and VEGF stimulated umbilical endothelial invasion, as well (9). In an immunohistological study, VEGF-B protein expression in alveoli, blood vessels, and type II pneumocytes of lungs exposed to chronic hypoxia was reported to have a significant amount of volume reduction

Table 1. The average staining intensity scores of VEGF in 0-h control, 6-h control, 6-h hypoxia, 12-h control, and 12-h hypoxia groups. Means with different superscripts in columns and rows differ significantly ($P < 0.01$). All data are shown as mean ± SD.

VEGF (average of staining scores)				
	0-h group (start)	6-h group	12-h group	P-value
Control	$3.4526^{a} \pm 0.81$	$2.5686^{bd} \pm 0.84$	$3.4364^{abc} \pm 0.65$	0.01
Hypoxia		$1.9656^{c} \pm 0.78$	$2.2654^{d} \pm 1.18$	0.01
P-value		0.01	0.01	

Table 2. The average measurements of medium of VEGF in 0-h control, 6-h control, 6-h hypoxia, 12-h control, and 12-h hypoxic groups from fibroblasts subjected to hypoxia. Means with different superscripts in columns and rows differ significantly ($P < 0.01$). All data are shown as mean ± SD.

VEGF (ng/mL)				
	0-h group (start)	6-h group	12-h group	P-value
Control	$1.20^{a} \pm 0.05$	$1.22^{a} \pm 0.10$	$1.18^{a} \pm 0.07$	NS
Hypoxia		$1.27^{a} \pm 0.09$	$1.18^{a} \pm 0.02$	NS
P value		NS	NS	

(24). In this immunocytochemical study presented here, VEGF containing 165, 189, and 121 amino acids (VEGF-A, -C, and -D) was used as antibody for the detection of isoenzymes and fibroblasts isolated from lung tissues of broiler chickens were seen to synthesize VEGF in hypoxic cell culture conditions. Broadly, the hypoxic groups of 6 and 12 h were found to have lower staining scores than the control group with fibroblast cells kept at normoxic conditions. In the 6-h hypoxic group, compared to the control, there was an approximately 1.3-fold decrease in staining score. In addition, there was a 1.5-fold lower staining score observed in the 12-h hypoxic group, suggesting that hypoxic stress caused the release of VEGF from fibroblasts into the cell culture media. Since immunocytochemical staining scores in the 6-h hypoxic group were lower than those at 12 h (secretion of VEGF to medium), it has been postulated that acute hypoxia may trigger VEGF more strongly than long-term hypoxia in lung fibroblasts since VEGF is secreted into the medium. This effect might have a role in edema formation via increasing permeability in the lung in in vivo conditions.

The results of VEGF expression may vary according to experimental models, procedure or technique, cell line types and how hypoxia was inducted, and the origin of sample of cells. The results, compared with the controls, also might show variability (such as high, low, or stable) (5,9,19,24). Hanaoka et al. (19) reported that the VEGF levels in edema fluid of the high-altitude pulmonary edema (HAPE) patients were significantly decreased at the beginning of the acute phase compared with the recovery phase. Moreover, the concentration of VEGF in the bronchoalveolar lavage fluid in HAPE patients was markedly depleted compared with that of controls (19). However, it was documented in another study that in lungs exposed to 5 h of hypoxia, VEGF in bronchoalveolar fluids was 1.5-fold increased compared with the controls (21). Walter et al. (5) detected that VEGF level increased in the arterial and mixed venous blood but no change was seen in the pulmonary capillary VEGF concentration taken from men at high altitudes. Karatolis et al. (25) reported that VEGF levels in pericardial effusion and serum were 85-fold higher compared with blood levels and these results indicate an increased local production within the pericardial cavity. Sands et al. (24) found that VEGF-A protein expression was not altered following 14 days of exposure to chronic hypoxia. The 6-h hypoxic group was 1.04-fold higher compared to its control. Steinbrech et al. (22) demonstrated that 6 h of hypoxia caused a 140% increase in VEGF mRNA expression in hypoxic dermal fibroblasts.

In this study, immunocytochemical and ELISA results support that there was a time-dependent correlation between decreasing immunohistochemical staining score and increasing ELISA measurement value in the 6-h hypoxic group. It was deduced that 6 h of acute hypoxia on lung fibroblasts was triggering for intracellular synthesis, medium secretion of VEGF, and edema formation via increasing permeability in the lungs. At a later 12-h stage, VEGF may participate in an active process of healing since VEGF has diverse roles in lung injury, contributing to lung edema during the acute stage of alveolar lung injury but promoting repair of the lungs during recovery (26).

Exposure to hypoxia is associated with increased expression of VEGF, contributing to decomposition of the basement membrane via induction of gelatinases (MMPs) and inhibition of tissue inhibitors of metalloproteinase. Therefore, damaged structural integrity of the alveolar/capillary barrier increases pulmonary vascular leakage and causes edema in hypoxic pulmonary hypertension (17,21,27). During edema formation, the relation with hypoxia and MMPs has been examined both in vivo (10) and in vitro (11). However, the mechanism by which hypoxia increases the vascular permeability and causes edema remains unclear in pulmonary hypertension syndrome in broiler poultry. Further studies are necessary to clarify the pathogenic role and cause of edema formation in lungs. The cooperative action of MMP activation and VEGF in production and edema should be the subject of another study.

In conclusion, the presence of VEGF was shown for the first time in cultured chicken lung fibroblasts from meat-type chickens. There was a significant decrease in immunocytochemical staining score but a slight increase in the amount of VEGF in culture media during hypoxic conditions. The results suggest that VEGF might have a role in edema formation while contributing to other factors via increasing permeability with a currently unknown mechanism in the lungs of ascitic meat-type chickens.

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