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VCAM-1 Increases Levels of HGF in Eosinophilic Chronic Rhinosinusitis Cell Culture

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Abstract. *Background: Eosinophilic chronic rhinosinusitis (ECRS) is one of the most common diseases worldwide. To date the underlying cause remains unclear and no drug has been accredited for first-line therapy. VCAM-1 has been reported to play a pivotal role in establishing ECRS. Other authors have reported that inflammatory cytokines may mediate changes in the underlying epithelium in the sinuses through hepatocyte growth factor HGF. In our study, the effect of VCAM-1 on HGF levels was investigated. Materials and Methods: ECRS cell cultures were incubated with VCAM-1 and HGF levels were determined after 16, 24, 48 and 72 hours. RT-PCR was enrolled to depict the HGF-RNA levels. Results: Sixteen hours of incubation showed 28.5 pg/ml HGF, whereas in the control 16.3 pg/ml was detectable. After 24 and 36 hours, 37 pg/ml and 43.5 pg/ml HGF were measured in the incubated cell cultures, respectively; 72 hours of incubation with VCAM-1 resulted in 50.6 pg/ml HGF, whereas 23.5 pg/ml was determined in the controls. The RT-PCR for HGF also showed increased concentration in the incubated cells after 72 hours. Conclusion: VCAM-1 induced an increase in levels of HGF in the ECRS cell cultures. The rising transcriptional activity was demonstrated by means of RT-PCR. The levels of HGF were within physiological ranges, suggesting that a misbalance between HGF and VCAM-1 resulted in the establishment of ECRS. Further experiments are necessary to reveal the role of HGF in the development of ECRS. This is the first report about the effect of VCAM-1 on growth factors in ECRS cell culture.*

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Chronic rhinosinusitis (CRS) is a significant health problem that affects approximately 15% of the general population and is still increasing in prevalence. CRS is defined as a disease of the nasal and paranasal sinus mucosa present for more than 3 months with mucosal changes ranging from inflammatory thickening to gross nasal polyps (1-3). CRS represents a heterogenous group of sinus disease and includes different disease entities. The pathogenesis of CRS is still unknown. The diagnosis is based on symptoms, duration of symptoms, clinical examination, nasal endoscopy and CT scan. The contributing factors comprise anatomical variants, atopy, acetylsalicylic acid intolerance and microbial factors (4). Recently, some authors have reported on fungal microorganisms in CRS and suggested them as the pathogen that initiates and maintains a chronic inflammatory process (5, 6). In the response to fungal pathogens, eosinophils and activated mast cells are involved (7, 8). The histological feature of CRS is a persistent underlying eosinophilic inflammation (9). Activated eosinophils contribute to polypoid sinusitis by the release of major basic protein (MBP) in the mucus, epithelial disruption, basement membrane denudation, and through the production of inflammatory cytokines (9-11). With regard to the pathogenesis, this new entity is called eosinophilic chronic rhinosinusitis (ECRS).

The endothelial adhesion molecule, vascular cell adhesion molecule (VCAM-1), plays an important role in eosinophilic migration into the inflamed airways (12-13). The expression of VCAM-1 is regulated transcriptionally by cytokines and mediators of inflammation, such as activator protein-1 (AP-1) and nuclear factor-kappaB (NF-κB) (14). Hosokawa *et al.* showed that transforming growth factor-β1 (TGF-β1) induced intercellular adhesion molecule-1 (ICAM-1) expression and down-regulated VCAM-1 induced expression of hepatocyte growth factor (HGF) (15). However, Spoelstra *et al.* reported that TGF-β1 did not influence ICAM-1 expression, but down-regulated VCAM-1 expression on lung fibroblasts (16). The influence of different cytokines

on ICAM-1 and VCAM-1 may be dependent on the source of fibroblasts. ICAM-1 and VCAM-1 expression on HGF induced by TNF-α was inhibited by mitogen-activated protein kinase (MAPK) inhibitors; however, MAPK inhibitors did not influence ICAM-1 or VCAM-1 expression on HGF (17).

HGF is one of the most important angiogenic factors and was initially discovered as a protein secreted by fibroblasts that causes epithelial cell mitogenesis, proliferation and morphogenesis (18, 19). In 1987, HGF was first described by Nakamura as a 100 kDa pleiotropic mitogenic growth factor for hepatocytes originally isolated from rat platelets (20). HGF's role in establishing angiogenesis, organ regeneration, development of the nervous system, and tumor invasion emphasizes its important role in physiological and pathological processes. HGF transduces signals *via* its receptor, c-Met, a transmembrane tyrosine kinase (21). Ueki *et al.* reported that HGF injections resulted in reduced expression of TGF-β1 in animal models (22). Hosokawa *et al.* discovered that HGF may be involved in the control of Th1/Th2 cells (15).

In the following experiments, we investigated the effect of VCAM-1 on ECRS cell cultures with regard to the expression of HGF. This is the first report about the influence of the Th-2 cytokine VCAM-1 on growth factors in ECRS.

Materials and Methods

Tissue collection and culture of human paranasal mucosa. All CRS cells were obtained from 6 patients suffering from CRS and undergoing functional endoscopic sinus surgery at the Department of Otorhinolaryngology at the University of Mannheim, Germany, in 2006. Prior to surgery, written consent was obtained from all patients to take tissue samples of the resected paranasal mucosa and turbinates. This study was approved by the Ethics Committee of the Faculty of Medicine, Mannheim, University of Heidelberg, Germany. Surgery was followed by pathological examination of the samples. The samples were diagnosed as eosinophilic and non-eosinophilic CRS depending on the amount of eosinophilic granulocytes in the samples. An epithelial culture for each sample of paranasal sinus mucosa and inferior nasal turbinate was established. After removal of connective tissue, the tissue specimens were cut into small pieces and incubated in trypsin solution (0.25% trypsin in phosphate-buffered saline, PBS) overnight at 4˚C. For primary culture of epithelial cells, the suspension was added onto mitomycin-treated (23.9 μM) human fibroblast monolayers and cultured in FAD2 medium (Dulbecco's modified Eagle's medium and Ham's F12 in a 3:1 ratio supplemented with fetal calf serum, adenine, insulin, triiodothyronine hydrocortisone, epidermal growth factor, cholera toxin and penicillin/streptomycin) at 37˚C in a 10% CO_2 atmosphere. On reaching subconfluency, the feeder layer was removed by incubation with 0.02% ethylenediamine tetraacetic acid (EDTA) in PBS for 4 min at 37˚C and the sinus epithelial cells were further cultured in keratinocyte growth medium (KGM, Clonetics, San Diego, CA, USA) without serum. Cells were passaged by trypsinization (0.1% trypsin and 0.02% EDTA dissolved in PBS, 5 min, 37˚C).

HGF-ELISA technique. The exact concentration of HGF was evaluated by using the Quantikine HGF Immunoassay from R&D Systems ((Minneapolis, MN, USA, cat-#: DHG 00). The assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for HGF was precoated onto a microplate. Standards and samples were pipetted into wells and HGF present was bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for HGF was added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of HGF bound in the initial step. The colour development was stopped and the intensity of the colour was measured. The exact concentration of HGF was determined following the basic principles using a standard curve. The cells were grown in 96-well plates with 12 strips of 8 walls coated with mouse antibody against HGF. After 8, 24, 48 and 72 hours of incubation with 100 ng/ml human recombinant VCAM-1 (catalog number 809-VR) from R&D Systems (Minneapolis, MN, USA), the expression of the HGF was analyzed in the supernatants of the treated and untreated cell cultures.

RT-PCR. To isolate RNA from the cells grown in monolayer, cells were directly lysed in the culture dish by the addition of 1 ml RNA-Clean (RNA-Clean System, AGS, Heidelberg, Germany). After addition of 0.2 ml chloroform per 2 ml of homogenate and centrifugation for 15 minutes at 12 g (4˚C), the aqueous phase was transferred to a fresh tube. After the addition of an equal volume of isopropanol and 12 g centrifugation for 15 minutes at 4˚C, the supernatant was removed from the RNA precipitate. The RNA pellet was washed twice with 70% ethanol by vortexing and subsequent centrifugation for 8 minutes at 7.5 g (4˚C). After drying the RNA pellet, it was dissolved in DEPC water. The RNA was reverse transcribed (StrataScript First-Strand Synthesis System; Stratagene, La Jolla, CA, USA) into cDNA using random-oligonucleotide primers. HGF mRNA levels were measured using RT-PCR (MMP-CytoXpress Multiplex PCR Kit, BioSource, San Francisco, CA, USA) according to the manufacturer's instructions. To fractionate the MPCR DNA products, MPCR products were mixed with 6X loading buffer and separated on a 2% agarose gel containing 0.5 mg/ml ethidium bromide, visualized with UV light and recorded using a CCD camera. To test the quality of the cDNA, the kit includes primers for GAPDH. Results were obtained in two independent experiments.

Western blot analysis. For Western blot analysis, the protein extracts of the cell culture were separated in a Tris-glycine gel (Invitrogen, Carlsbad, CA, USA) and transferred onto a polyvinylidene difluoride membrane. Western blot analysis was performed as described elsewhere (25).

Results

After 16 hours of incubation, 28.5 pg/ml HGF was determined in the supernatants of the treated cell cultures, whereas in the controls 16.3 pg/ml was detectable. After 24 and 36 hours, 37 pg/ml and 43.5 pg/ml HGF were measured in the treated cell cultures respectively. However, the untreated cells showed 19.4 pg/ml and 22

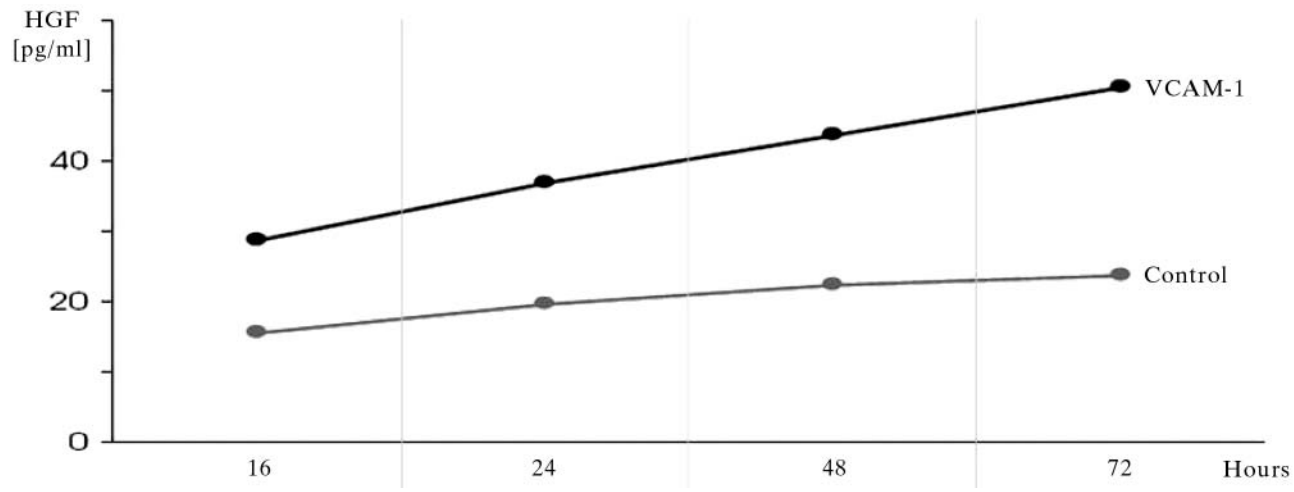

Figure 1. *Incubation of the CRS cells with VCAM-1 and levels of HGF after 72 hours.*

pg/ml HGF. Seventy-two hours of incubation with VCAM-1 resulted in 50.6 pg/ml HGF whereas in the controls 23.5 pg/ml was determined.

The RT-PCR also showed increased concentration of HGF after incubation with VCAM-1 (Figure 1). Western blot analysis confirmed the rt-PCR results (Figure 2).

In summary, VCAM-1 resulted in increased levels of HGF in the treated cell cultures. The histological morphology of the cells was not changed. The proliferation assay showed no significant difference between treated and untreated cell cultures.

Discussion

VCAM-1 and various different interleukins have been reported to be the most relevant pathogenetic proteins resulting in ECRS (24). Hosokawa *et al.* reported that in inflamed epithelial mucosa VCAM-1 expression was induced by HGF. Different authors have shown that HGF up-regulates the expression of other inflammatory and growth proteins *via* the receptor c-Met, which is a tyrosine kinase receptor (20, 23-25). HGF was shown as an anti-organotoxic agent in several organs. Furthermore, HGF is synthesized by various cell types, including fibroblasts, macrophages and other inflammatory cells, smooth muscle cells, and epithelial cells (26-27). However, regarding clinical relevance for possible therapeutic strategies, the effect of VCAM-1 on HGF levels in ECRS is most important. Therefore, ECRS cell cultures were incubated with VCAM-1 and HGF levels were determined after a period of up to 72 hours.

The significant difference between the treated cells and controls was shown in the rising levels of HGF. Regarding the experiments of Hosokawa *et al.*, HGF and VCAM-1 seem to partly have a synergistic effect on each other. However, Min *et al.* reported that HGF counteracted VEGF-induced expression of VCAM-1 on endothelial cells at the transcriptional level (28). They also found out that HGF inhibited VEGF-activation of transcription factors that are important for the expression of inflammatory genes on the vascular wall (28). Ito *et al.* illustrated that HGF resulted in the reduction of both goblet cell hyperplasia and mucus hyperproduction in the lungs (29).

The different results demonstrate HGF to be a biphasic protein. On the one hand, HGF up-regulated the expression of the inflammatory protein VCAM-1; on the other hand, HGF reduced inflammation in epithelial tissue and acted as a protective agent. In our experiment HGF, was increased by VCAM-1 which, besides synergistic effects, assigns HGF a protective characteristic. The increase of HGF levels seems to regulate the level of inflammation in tissue. Counteracting inflammation and increasing VCAM-1 could result in a "status quo". In ECRS, a misbalance between HGF and VCAM-1 expression could be the major reason for the uncontrolled growth of polyps.

Alongside motogenic, angiogenic, and oncogenic features, HGF is also characterized as mitogenic and morphogenic (20). It has been reported that the morphology of cells was altered after up-regulated expression of HGF (20, 23). In our experiments, no alteration of the histological morphology and unmodified proliferation assay of the ECRS cells was observed. The expression of HGF ranged between 28 and 51 pg/ml and between 15 and 23 pg/ml in the ECRS cultures and control respectively. With regard to previous experiments, Peruzzi and Bottaro reviewed levels above 200 pg/ml up to 3000 pg/ml as mitogenic, morphogenic, and oncogenic concentrations (30). Although VCAM-1 significantly

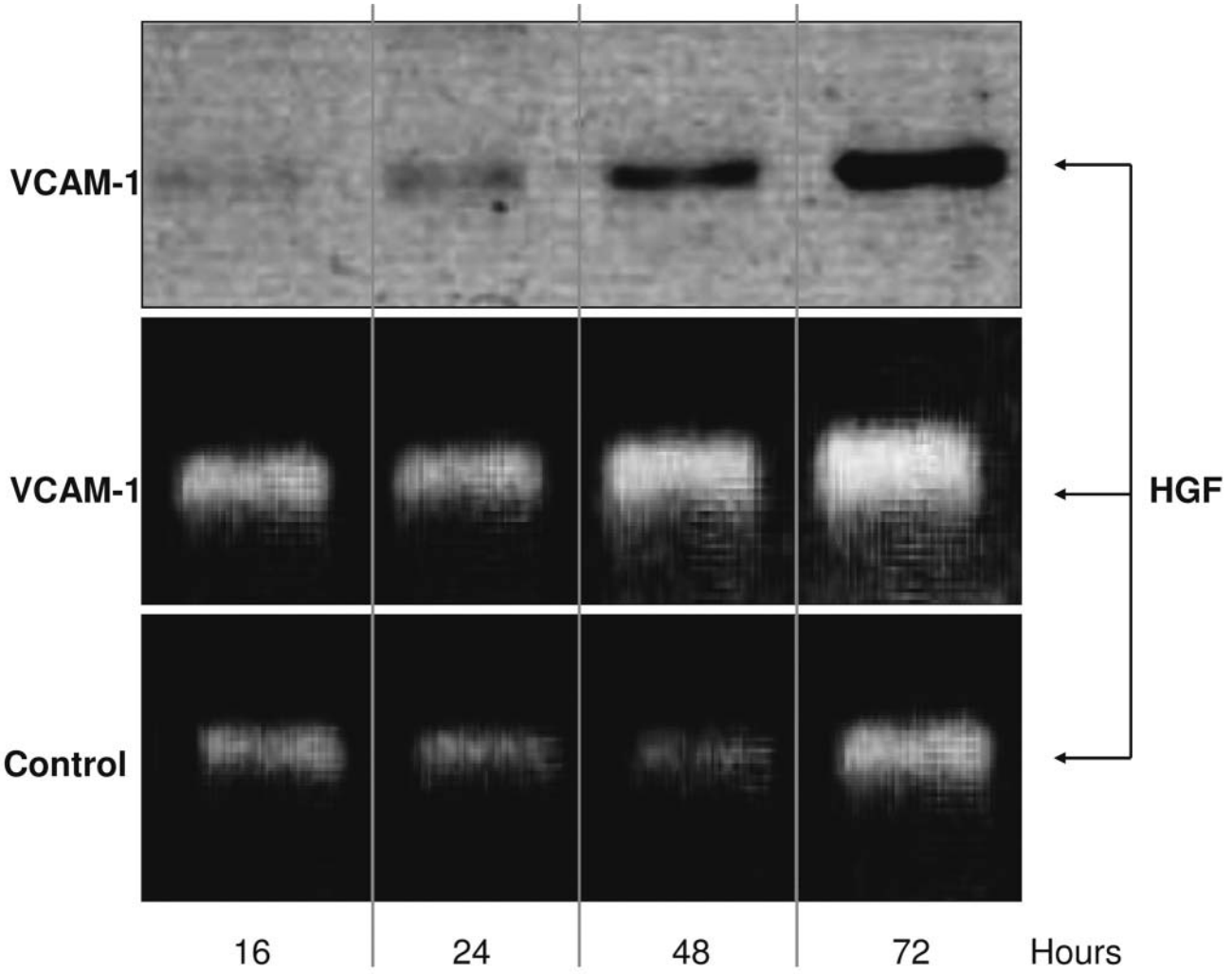

Figure 2. *RT-PCR and Western blot analysis showing increasing HGF in the cultures of the treated CRS cells after incubation of the CRS with VCAM-1.*

altered the expression of HGF in the ECRS cell culture, the levels were significantly below the mitogenic and morphogenic levels suggested elsewhere. In this study, after 72 hours of incubation, the determined concentration of HGF was obviously still within physiological limits. Thus, our hypothesis of misbalanced regulation between VCAM-1 and HGF was supported by our experiments.

It was also interesting to investigate whether VCAM-1 up-regulated HGF *via* depleting intracellular storage of HGF or by altering transcriptional levels. The RT-PCR showed that the level of HGF RNA increased with the duration of the incubation. According to our results, VCAM-1 seemed to have a transcriptional effect on the expression of HGF. Cytokine-dependent VCAM-1 induction is regulated at the gene level by the activity of transcription factors such as NF-κB, AP-1, specificity protein-1 (SP-1), Interferon regulatory factor-1 (IRF-1), and GATA (31). However, there has been no report about transcriptional activity of VCAM-1 with regard to growth factors, such as HGF.

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