

The presence of high level soluble herpes virus entry mediator in sera of gastric cancer patients

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<http://dx.doi.org/10.3858/emm.2012.44.2.010>

Accepted 23 November 2011

Available Online 24 November 2011

Abbreviations: DCF-DA, 2',7'-dichlorofluorescein diacetate; HVEM, herpes virus entry mediator; LIGHT, homologous to lymphotoxins, shows inducible expression and competes with herpes simplex virus glycoprotein D for herpes virus entry mediator (TNF Ligand Superfamily, Member 14; TNFSF14); rhLIGHT, recombinant human LIGHT

Abstract

The development of gastric cancer (GC) is closely related to chronic inflammation caused by *Helicobacter pylori* infection, and herpes virus entry mediator (HVEM) is a receptor expressed on the surface of leukocytes that mediates potent inflammatory responses in animal models. However, the role of HVEM in human GC has not been studied. Previously, we showed that the interaction of HVEM on human leukocytes with its ligand LIGHT induces intracellular calcium mobi-

lization, which results in inflammatory responses including induction of proinflammatory cytokine production and anti-bacterial activities. In this study, we report that leukocytes from GC patients express lower levels of membrane HVEM (mHVEM) and have lower LIGHT-induced bactericidal activities than those from healthy controls (HC). In contrast, levels of soluble HVEM (sHVEM) in the sera of GC patients were significantly higher than in those of HC. We found that monocyte membrane-bound HVEM is released into the medium when cells are activated by proinflammatory cytokines such as TNF- α and IL-8, which are elevated in the sera of GC patients. mHVEM level dropped in parallel with the release of sHVEM, and release was completely blocked by the metalloprotease inhibitor, GM6001. We also found that the low level of mHVEM on GC patient leukocytes was correlated with low LIGHT-induced bactericidal activities against *H. pylori* and *S. aureus* and production of reactive oxygen species. Our results indicate that mHVEM on leukocytes and sHVEM in sera may contribute to the development and/or progression of GC.

Keywords: cytokines; inflammation; monocytes; receptors, tumor necrosis factor, member 14; stomach neoplasms; TNFSF14 protein, human

Introduction

It is now well established that inflammation plays critical roles in the development of cancer and metastases. Inflammation is caused by many agents including microbial infections, autoimmune diseases and obesity, and involves complex interactions between proinflammatory cytokines and cells (Mantovani *et al.*, 2008). Tumor necrosis factor α (TNF- α) is one of the most potent proinflammatory cytokines and is involved in the pathogenesis of many inflammatory diseases and tumors (Balkwill, 2009). Therefore, TNF- α and its receptors have long been targets in the treatment of a variety of human diseases. For example, some patients with inflammatory diseases are helped by preventing TNF- α from binding to TNF receptors

(TNFR). However, some patients are resistant to treatment by TNF- α blockade, implying that the role of TNF- α is not universal in human diseases (Zhu *et al.*, 2010; Benucci *et al.*, 2011). The TNF superfamily (TNFSF) and the TNFR superfamily (TNFRSF) each contain several members, and TNFSF-TNFRSF interactions regulate a number of normal and pathological processes, including immune responses, hematopoiesis, morphogenesis, tumorigenesis, septic shock, and inflammatory disease (Aggarwal, 2003). It is likely that additional TNFSF-TNFRSF interactions are involved in inflammation-induced human diseases including those resistant to TNF- α blockade.

Herpes virus entry mediator (HVEM), a TNFRSF protein, is widely expressed on the plasma membranes of hematopoietic lineage cells, such as T cells, B cells, natural killer cells, monocytes, neutrophils and dendritic cells (Kwon *et al.*, 1997; Heo *et al.*, 2006). HVEM has multiple ligands and is unique among TNFRSF proteins in binding to both TNFSF and immunoglobulin superfamily (IGSF) proteins. In addition to the TNFSF proteins LIGHT (homologous to lymphotoxins, shows inducible expression and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes) and lymphotoxin α , the IGSF proteins B and T lymphocyte attenuator (BTLA) and CD160 bind to HVEM (Shui *et al.*, 2011), as does herpes simplex virus glycoprotein D. When LIGHT binds to HVEM, positive co-stimulatory signals are delivered through both LIGHT and HVEM that result in potent leukocyte inflammatory responses, such as the production of inflammatory cytokines and ROS. On the other hand, binding of HVEM to BTLA delivers inhibitory signals by recruiting tyrosine phosphatase, and these signals lead to suppression of immune responses. Therefore, HVEM can act as a molecular switch, either activating or inhibiting immune responses. In this regard, many studies have focused on the HVEM/LIGHT/BTLA pathway in animal models of tumors, autoimmune disease, infection and transplantation (del Rio *et al.*, 2010). However, few studies have investigated the roles of HVEM and/or LIGHT in human diseases including cancer.

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide, and approximately 700,000 people succumb to it each year. It has been shown that *Helicobacter pylori*, by which half the world's population is infected, is closely associated with the development of GC, suggesting that inflammation is involved in that process (Wroblewski *et al.*, 2010). In a previous study, we showed that HVEM induces potent inflammatory responses by neutrophils and monocytes (Heo *et*

Table 1. Gastric Cancer Patient Characteristics

Clinopathological Characteristics	No
Age	
< 60	5
> 60	10
Sex	
Male	6
Female	9
Cancer stage ^a	
IA	5
IB	3
IIA	1
IIB	3
IIIA	1
IIIC	2
Gastritis ^b	
Positive	6
Negative	9
<i>H. pylori</i> infection ^c	
Positive	1
Negative	3
ND ^d	11

^aTNM stage; ^bdetermined by endoscopy; ^cdetermined by CLO test; ^dnot determined

et al., 2006). Therefore, we hypothesized that leukocytes from GC patients might have altered levels of HVEM and HVEM-mediated immune responses. Here we report that the leukocytes of GC patients contain significantly lower levels of HVEM than those of healthy controls, and GC patients have higher soluble HVEM concentrations in their sera than do healthy controls. Surprisingly, we also found that membrane-bound HVEM is shed when cells are activated by a variety of stimuli including the proinflammatory cytokines IL-8 and TNF- α , whose concentrations are highly elevated in the sera of GC patients.

Results

Patient characteristics

A summary of the enrolled patients is presented in Table 1. Blood samples from 15 patients with GC and 22 healthy controls (HC) were investigated. Median age of GC and HC was 59.5 ± 11.4 and 53.9 ± 2.7 years, respectively.

GC patient leukocytes have reduced levels of membrane HVEM (mHVEM)

It has previously been demonstrated by this research group that HVEM was expressed on the membranes of T-cells, B-cells, monocytes and neutrophils isolated from human peripheral blood (Heo *et al.*, 2006). Of those, monocytes and neutrophils were the most highly HVEM-expressing cells. HVEM expression on the membranes of monocytes and neutrophils in GC patients and healthy controls (HC) were compared by staining cells with fluorescence-labeled anti-HVEM antibody and examining them by FACS. As shown in Figure 1A, GC patient monocytes had significantly lower levels of mHVEM than HC monocytes. When analyzed as the percentage of HVEM-expressing cells, $94.6 \pm 3.5\%$ of HC monocytes expressed HVEM, compared with only $49.1 \pm 12.8\%$ of GC patient monocytes (Figure 1B). The percentage of HVEM-expressing neutrophils was also substantially lower in GC patients than in HC ($37.0 \pm 9.5\%$ vs $67.7 \pm 10.7\%$).

It has been shown that soluble HVEM (sHVEM) is elevated in patients with allergic and autoimmune diseases such as allergic asthma, atopic dermatitis and rheumatoid arthritis (Jung *et al.*, 2003). However, sHVEM levels in cancer patients has not been reported. Blood levels of sHVEM in HC and GC patients were determined by ELISA as described in Methods. In sharp contrast with the results for membrane-bound HVEM, sHVEM levels in GC patient sera were more than 3-fold higher than in HC (mean \pm SD; 748 ± 461 and $2,619 \pm 1,195$ pg/ml for HC and GC patients, respectively ($P < 0.01$)) (Figure 1C).

High levels of proinflammatory cytokines in GC patient sera

Cytokines are major regulators of immune cells affecting receptor expression and cell activation. It has been reported that sera of advanced stage GC patients contain elevated levels of inflammatory cytokines (Tsujiimoto *et al.*, 2010). Serum cytokine levels were measured to investigate mechanisms underlying altered expression of leukocyte mHVEM and sHVEM in GC patients. As shown in Figure 2, TNF- α , IL-1 β , IL-6, and IL-8 levels were significantly higher in the sera of GC patients than in those of HC. In contrast, IL-2, IL-4, IL-10 and IFN- γ levels were significantly lower, while IL-12 levels did not differ significantly in GC patients and HC.

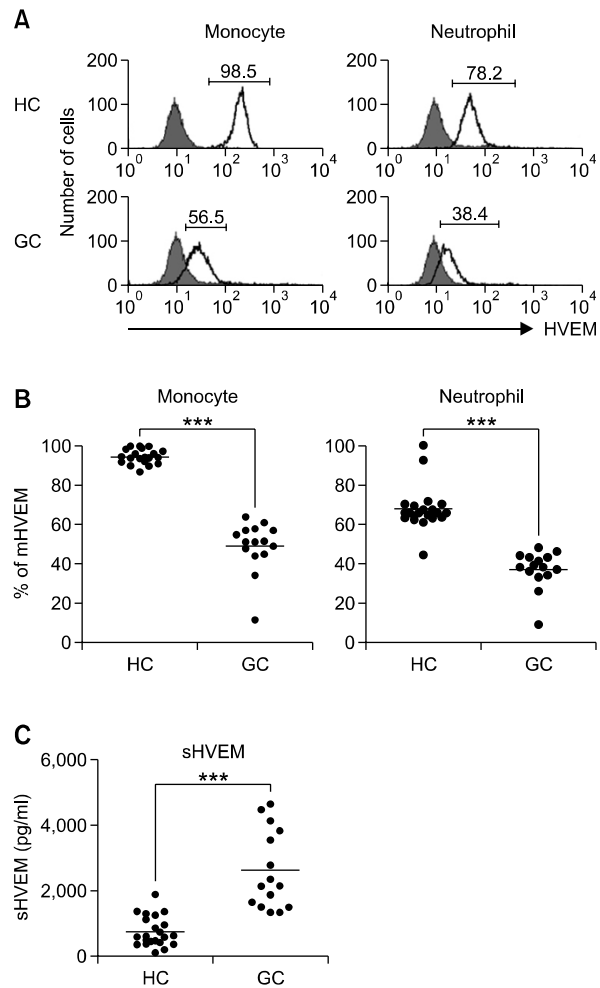


Figure 1. GC patients have lower mHVEM levels in their leukocytes and higher sHVEM levels in their sera than healthy controls. (A) Histogram of mHVEM expression in monocytes and neutrophils. Monocytes and neutrophils of GC patients and healthy controls were purified as described in Methods. Cells were stained with FITC-conjugated anti-HVEM antibody (open histogram) or isotype control (filled histogram) and analyzed by FACS. Results shown are representative of three experiments. (B) Percentages of HVEM-expressing monocytes (left panel) and neutrophils (right panel) in GC patients and HC were determined as in A. Each dot represents the % of HVEM expressing cells in a sample. Horizontal bars depict means. (C) sHVEM levels in sera of GC patients and HC. Concentrations of sHVEM in sera were determined by ELISA as described in Methods. Horizontal bars depict means. Each datum shown is the mean of triplicate measurements. *** $P < 0.001$ compared with control groups.

Stimulation of monocytes induces release of sHVEM with a concurrent decrease of mHVEM

It has been shown that membrane-associated HVEM on T lymphocytes is downregulated when the cells are activated (Morel *et al.*, 2000). However, the regulation of HVEM expression on leukocytes has not been reported. It was hypothesized that activation of leukocytes by inflammatory stimulants might induce mHVEM release into the extracellular

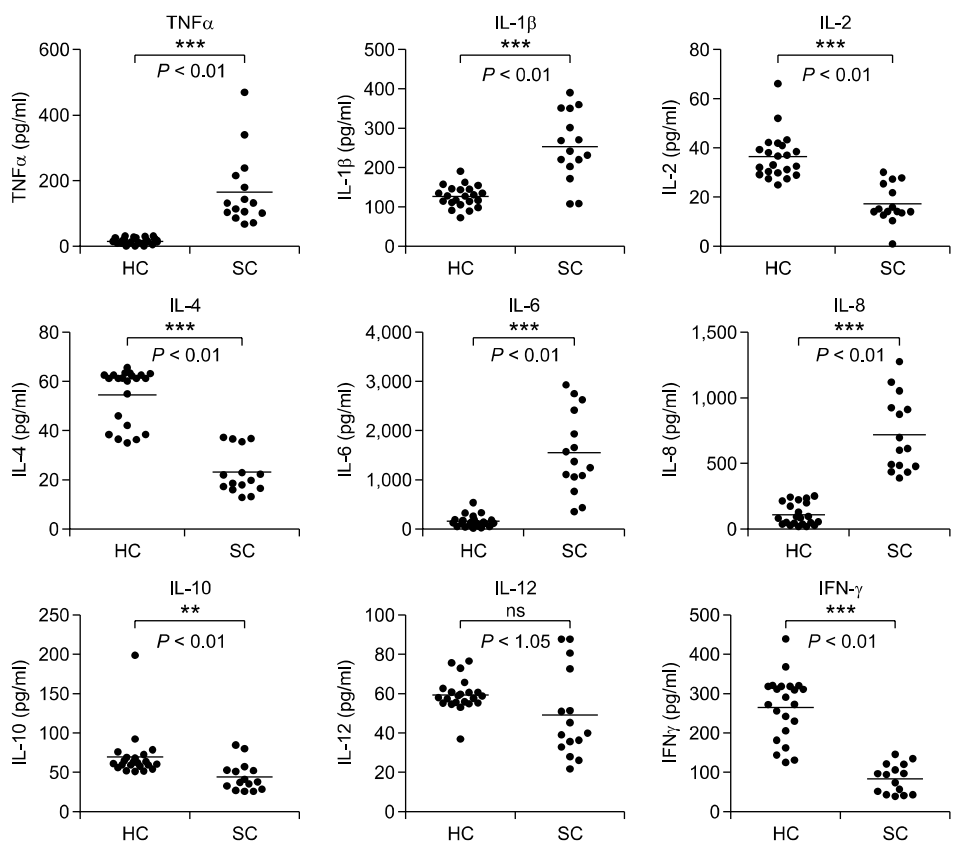


Figure 2. Cytokine levels in sera of GC patients and HC. Peripheral blood from GC patients and HC was coagulated to obtain sera. Cytokine concentrations in sera were determined with ELISA kits (Endogen, MA). Each datum is the mean of triplicate measurements. Horizontal bars depict means. ** $P < 0.01$ and *** $P < 0.001$ compared with HC. ns, not significant.

space and lead to the high levels of sHVEM observed in GC patient sera. To test this idea, monocytes were cultured in the presence of a variety of cell activating agents, such as LPS, PHA, calcium ionophore A23187 and PMA for 24 hr and measured levels of HVEM on cell membranes (mHVEM), and of sHVEM in culture supernatants. As shown in Figure 3A, all of those stimulants decreased mHVEM and increased sHVEM, indicating that non-specific activation of monocytes induces release of mHVEM into the culture medium. The specific HVEM ligand, rhLIGHT, also dose-dependently decreased mHVEM and increased sHVEM (Figure 3B). Since GC patients have high levels of systemic inflammatory cytokines, such as TNF- α and IL-8 (Figure 2), it was investigated whether cytokines also affected mHVEM expression on monocytes and sHVEM levels. As expected, both cytokines decreased mHVEM and increased sHVEM in the culture medium (Figure 3C).

The release of extracellular protein domains, called ectodomain shedding, is now recognized as a general mechanism for regulating the function of transmembrane proteins. Zinc-based metalloproteases, such as metalloprotease disintegrins and matrix metalloproteases, mediate the shedding of a variety of membrane receptors and cytokines

(Arribas and Borroto, 2002). To see whether metalloproteases are involved in the activation-induced release of sHVEM in leukocytes, the effect of GM6001, which inhibits a wide spectrum of metalloproteases, was studied. Freshly isolated human monocytes from HC were stimulated with rhLIGHT, rhIL-8 or rhTNF- α in the presence of GM6001 for 24 hr and mHVEM and sHVEM levels in the culture medium were determined. It was found that GM6001 progressively inhibited the rhLIGHT-induced decrease of mHVEM and rhLIGHT-mediated increase of sHVEM (Figure 4A). Release of monocyte mHVEM by rhIL-8 and rhTNF- α was also inhibited by GM6001 (Figure 4B). These results indicate that when monocytes are activated, mHVEM is cleaved by a metalloprotease and released into the culture medium.

GC patient leukocytes have lower HVEM-mediated bactericidal activities and produce less ROS than leukocytes of healthy controls

Our finding that mHVEM is low on GC leukocytes prompted a test of whether altered mHVEM level affected HVEM-mediated leukocyte function. It was previously demonstrated by this research group that binding of recombinant human LIGHT (rhLIGHT)

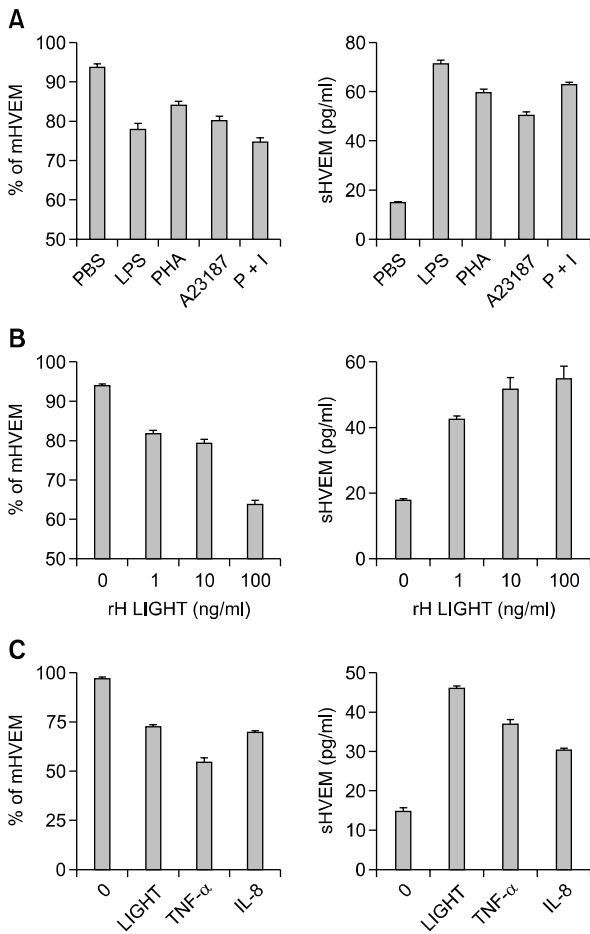


Figure 3. Stimulation of monocytes decreases mHVEM and increases sHVEM. (A) Monocytes from HC were cultured in RPMI1640 + 10% FBS in the presence of 100 ng/ml LPS, 1 µg/ml PHA, 10 nM A23187, or 50 ng/ml PMA and 200 ng/ml ionomycin. After 24 hr, cells and culture supernatants were harvested. Cells were stained with anti-HVEM-FITC mAbs or isotype control mAbs (Mouse IgG1) and analyzed by flow cytometry, and percentages of mHVEM-expressing cells are shown (left panel). Concentrations of sHVEM in culture supernatants were analyzed by ELISA (right panel). (B) Monocytes were cultured in the presence of 0, 1, 10 or 100 ng/ml rhLIGHT for 24 hr, and the % of mHVEM-expressing cells and sHVEM concentrations in culture supernatants were determined as in (A). (C) Monocytes were cultured with 100 ng/ml of rhLIGHT, rhTNF-α or rhIL-8 for 24 hr, and the % of mHVEM expressing cells and sHVEM concentrations in culture supernatants were determined as in (A). Results shown are representative of at least three independent experiments and are means ± SEM of triplicates. ****P* < 0.001 compared with rhLIGHT- alone group. mHVEM, membrane bound HVEM; sHVEM, soluble HVEM.

protein to HVEM in leukocytes activates cytoplasmic phospholipase γ1, which results in ROS generation and enhancement of bactericidal activities (Heo *et al.*, 2006, 2007). Neutrophils and monocytes from HC and GC patients were isolated, allowed to ingest *S. aureus* and incubated with 1, 10, and 100 ng/ml of rhLIGHT for 10 min; then the number of viable bacteria remaining in the cells

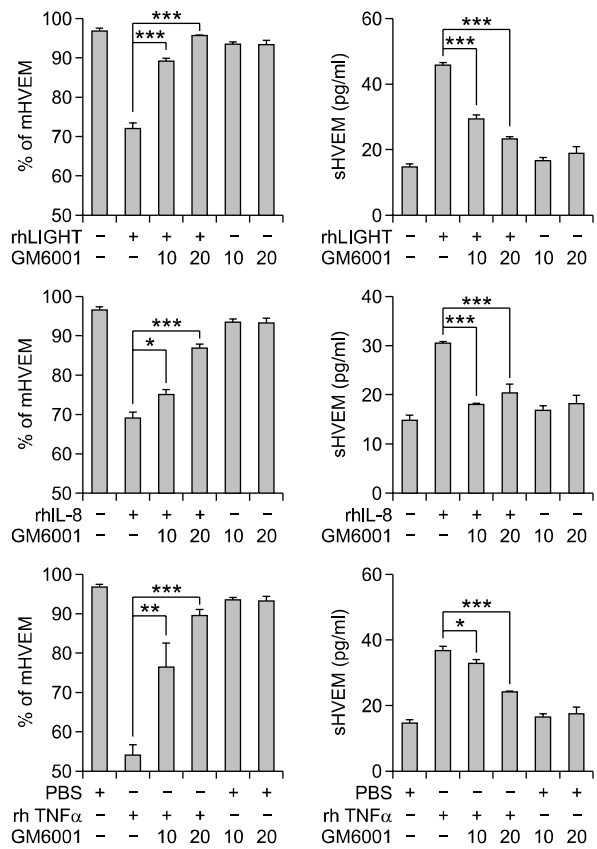


Figure 4. Metalloprotease inhibitor GM6001 blocks the activation-induced decrease of mHVEM and increase of sHVEM in cultured monocytes. Cells purified from HC were cultured in the presence of the indicated agonists (10 ng/ml) and/or GM6001 (10 or 20 µM), and the % of mHVEM expressing cells and sHVEM concentrations in culture supernatants were determined as in Figure 3. Results shown are representative of at least three independent experiments and are means ± SEM of triplicates. ****P* < 0.001, ***P* < 0.01 and, **P* < 0.05 compared with rhLIGHT alone group. mHVEM, membrane bound HVEM; sHVEM, soluble HVEM.

was determined by plating serial tenfold dilutions of cell lysates on agar plates. Figure 5A shows that the bactericidal activity of HC monocytes and neutrophils was dose-dependently increased by rhLIGHT. The bactericidal activities of GC patient monocytes and neutrophils were also increased by rhLIGHT but to a lesser extent. Heat-inactivated rhLIGHT did not enhance the bacterial killing activity of monocytes and neutrophils, indicating that there was no contamination with endotoxins (Figure 5A).

Next, rhLIGHT-induced bactericidal activities of GC patient monocytes and neutrophils against *H. pylori*, which is considered as a major agent developing gastric cancer, was determined. As shown in Figure 5B, *H. pylori* killing activities of GC patient monocytes and neutrophils were significantly lower

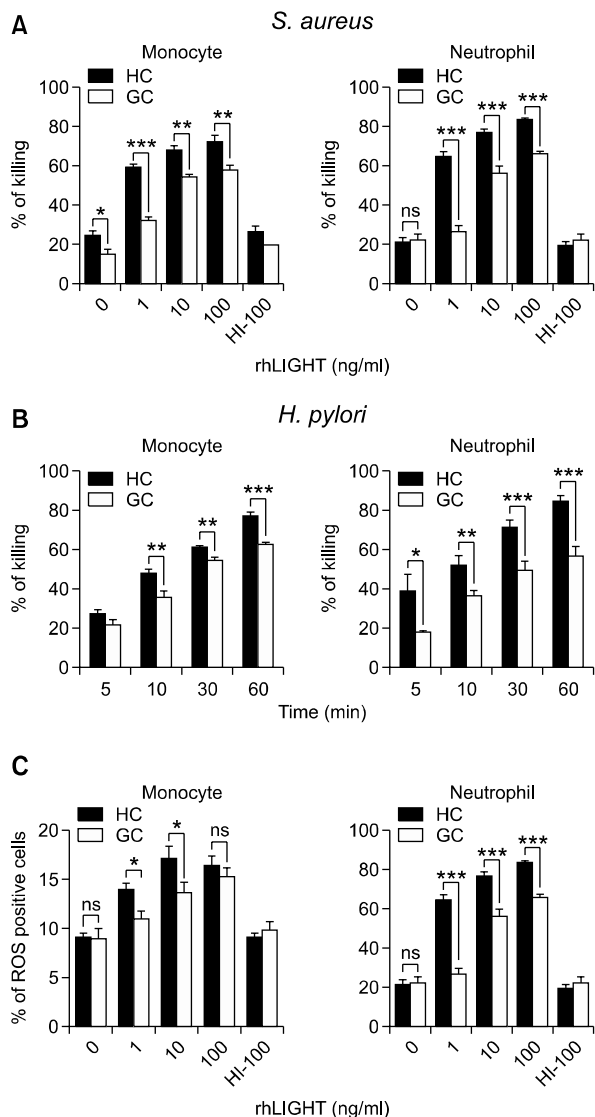


Figure 5. HVEM-mediated bacterial killing and ROS production by GC patient leukocytes are lower than by HC leukocytes. Monocytes and neutrophils of GC patients and healthy controls were purified and incubated with opsonized *S. aureus* or *H. pylori* for 3 min. After washing to remove non-ingested bacteria, (A) cells were incubated for 10 min with indicated concentrations of rhLIGHT or (B) cells were treated with 10 ng/ml of rhLIGHT for indicated times. After incubation, cells were lysed in distilled water, and numbers of viable bacteria per million cells were determined by plating on agar. (C) HVEM-mediated ROS production. Cells were stained with the ROS-sensitive fluorescent dye, DCF-DA, and cultured with 0, 1, 10 or 100 ng/ml of rhLIGHT. After 45 min, they were harvested and percentages of ROS-positive cells were assessed by flow cytometry. Filled bar, healthy controls; open bar, GC patients. HI-100, heat-inactivated rhLIGHT 100 ng/ml. Results shown are representative of at least three independent experiments from different blood donors and are means \pm SEM. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ compared with control group.

than those of HC cells. These results indicate that GC patient leukocytes have reduced activity of HVEM-mediated clearance of *H. pylori* infection in

GC patients, which might aggravate inflammation associated with GC development.

ROS, which are produced by activated, phagosome-bound NADPH oxidases, are major component of the bactericidal activity of monocytes and neutrophils and the rhLIGHT-HVEM interaction stimulates ROS production in human monocytes and neutrophils (Segal, 2005). rhLIGHT-induced ROS generation was compared in the leukocytes of GC patients and HC. Cells were stained with the ROS-sensitive fluorescent dye DCF-DA and incubated with rhLIGHT. They were then harvested, and fluorescent cells were detected by flow cytometry. As shown in Figure 5B, exposure of HC monocytes and neutrophils to rhLIGHT (1 and 10 ng/ml) stimulated ROS production, but 100 ng/ml rhLIGHT had less effect. rhLIGHT treatment of GC patient monocytes and neutrophils also increased ROS production. However, the % of ROS positive GC patient leukocytes was significantly lower than in HC leukocytes when compared at the same rhLIGHT concentrations, except for neutrophils treated with 100 ng/ml of rhLIGHT, which was maximally effective in stimulating ROS production. These results together with our previous finding that rhLIGHT-mediated bactericidal activity in leukocytes is dependent on ROS generation (Heo *et al.*, 2006), suggest that mHVEM levels on leukocytes influence HVEM-mediated leukocyte functions.

Discussion

Ectodomain shedding regulates the activity of a number of transmembrane proteins. Several physiological agents, such as chemokines, cytokines, growth factors and non-specific agonists such as PMA, have been shown to induce ectodomain shedding (Arribas and Borroto, 2002). TNFSF and TNFRSF proteins have also been shown to be regulated by ectodomain shedding. For example, TNF- α is synthesized in a transmembrane form, proTNF- α , and cleavage by TNF- α converting enzyme (TACE; also known as ADAM17), ADAM10 or MMP-7, releases the receptor-binding domain into the extracellular space (Wong *et al.*, 1989; Hikita *et al.*, 2009). Ectodomain shedding of HVEM has not been previously reported. Our earlier finding that patients with autoimmune disease have high levels of sHVEM in their sera is the first report of the existence of sHVEM (Jung *et al.*, 2003). We observed that levels of sHVEM in sera of patients with inflammatory diseases, such as rheumatoid arthritis, atopic dermatitis, psoriasis, systemic lupus erythematosus, Bechet's disease, allergic asthma or pneumonia, were 2- to 5-fold higher

than in sera of healthy controls. In the present study, we found that GC patients have roughly 3-fold higher levels of sHVEM levels in their sera than in age-matched healthy controls. Although we did not demonstrate cleavage of HVEM, several observations suggest that the sHVEM is produced by ectodomain shedding rather than by simple secretion. The primary feature of ectodomain shedding is that it is induced by non-specific agents such as PMA and many agonists (Arribas and Borroto, 2002), and we showed that the same was true for the increase of sHVEM and decrease of mHVEM in cultured monocytes (Figure 3A). Second, GC patients have high levels of inflammatory cytokines (Figure 2) and these were shown to induce the increase of sHVEM and decrease of mHVEM in cultured monocytes (Figure 3C). Lastly, inhibition of a metalloprotease known to cause ectodomain shedding also inhibited the release of sHVEM by cultured monocytes (Figure 4).

The soluble domains of receptors generated by shedding can regulate ligand functions by inhibiting or promoting the formation of active signaling complexes (Rose-John and Heinrich, 1994). Furthermore, transmembrane and cytoplasmic domains that remain bound to the cell following receptor ectodomain shedding have been shown in some cases to have autonomous signaling abilities (Fortini, 2001). At present, the roles of sHVEM and the transmembrane/cytoplasmic domain of HVEM are completely unknown, although the signaling pathway of mHVEM is well defined: when LIGHT binds to mHVEM, there is intracellular mobilization of calcium ion, and binding of adaptor proteins, referred to as TNFR-associated factors, to the cytoplasmic domain of HVEM leads to NF κ B activation (Wallach *et al.*, 1999; Heo *et al.*, 2007). This process can result in costimulation of T cells (Tamada *et al.*, 2000), induction of dendritic cell maturation (Zou *et al.*, 2004), stimulation of Ig production by B-cells (Duhon *et al.*, 2004), NK cell activation (Fan *et al.*, 2006) and enhancement of the anti-bacterial activities of leukocytes (Heo *et al.*, 2006). Researchers have reported that administration of an HVEM-Ig fusion protein consisting of the soluble extracellular domain of HVEM linked to the immunoglobulin Fc region affects HVEM-associated physiological functions such as mice immune responses to bacterial infections, the severity of graft-versus-host disease, collagen-induced arthritis, and cardiac allograft rejection (Ye *et al.*, 2002; Pierer *et al.*, 2009; Brown *et al.*, 2010). Therefore, there is a good possibility that the sHVEM generated by ectodomain shedding has an *in vivo* role. Because HVEM has multiple ligands, such as LIGHT, BTLA and CD160, and LIGHT can bind to LT β R in addition to HVEM,

sHVEM-mediated affects on molecular interactions could be very complex. Such interactions might include HVEM-LIGHT, HVEM-BTLA, HVEM-CD160 and LIGHT-LT β R interactions. Identification of the role of the sHVEM might suggest ways to interfere with gastric cancer development.

The immune system can recognize and eliminate transformed tumor cells in a process named cancer immunosurveillance. However, the immune system can also promote tumor progression. These dual anti-tumor and pro-tumor actions of the immune system are now referred to as cancer immunoediting (Schreiber *et al.*, 2011). Proinflammatory cytokines have major effects on the pro-tumor activities of the immune system. For example, TNF- α produced by tumor cells and leukocytes promotes the survival and proliferation of malignant cells, stimulates angiogenesis and metastasis, subverts adaptive immunity, and alters responses to hormones and chemotherapy (Balkwill, 2009). There is good evidence that immune cell-mediated inflammation also regulates the development and progression of gastric cancers. Thus, the major cause of gastric cancer is *H. pylori* infection, which is now recognized as an infectious disease because chronic *H. pylori* infection is so closely related to GC development (Piazuelo *et al.*, 2010). Among 1526 Japanese patients who had gastric diseases, gastric cancer developed only in those patients infected by *H. pylori* (Uemura *et al.*, 2001). Moreover, polymorphisms of several inflammatory cytokine genes, such as those for TNF- α and IL-1, affect GC risk (Crusius *et al.*, 2008; Seo *et al.*, 2010). In addition, GC patients have high levels of inflammatory cytokines in their sera as shown in this study (Figure 2) and by others (Tsujimoto *et al.*, 2010). Although the role of HVEM in gastric cancer in association with inflammation is unknown, our finding that GC patient leukocytes have low levels of mHVEM but produce high levels of sHVEM adds to the evidence that GC is closely associated with inflammatory processes. Furthermore, it is noteworthy that in addition to gastric cancer, many cancers, such as hepatocellular carcinoma, B-cell lymphoma and cervical carcinoma are caused by infectious agents, which are closely associated with chronic inflammation (Coussens and Werb, 2002). Therefore, there is a substantial possibility that those cancer patients also have altered levels of sHVEM and mHVEM in their sera and leukocytes. We are currently investigating the level of sHVEM and mHVEM in various tumor patients.

Methods

Reagents

Recombinant human LIGHT (rhLIGHT) was purchased from R&D Systems (Minneapolis, MN), and diluted in 0.1% BSA-PBS buffer. According to the suppliers, the endotoxin concentration was less than 0.1 ng per 1 µg of protein as determined by the LDL method. Recombinant human IL-8 (rhIL-8), rhTNF-α and rhIL-2 proteins were purchased from Endogen (MA, USA). Polymorphprep™ for purifying neutrophils was from Nycomed Pharma (Oslo, Norway) and RPMI 1640 and Hank's balanced salt solution (HBSS), from GibcoBRL (Grand Island, N.Y.). ELISA kits for cytokines were purchased from Endogen (Thermo Fisher Scientific, Rockford, IL). All other reagents were from Sigma (St. Louis, MO). For heat inactivation, proteins were incubated for 20 min at 80°C.

Test samples

Patients with GC and healthy volunteers were enrolled after obtaining informed consent, and the study was approved by the institutional review board of Ulsan University Hospital (#09-30). Blood samples were obtained once from healthy volunteers and were collected from GC patients one day prior to the first chemotherapy treatment at a time when they were chemotherapy-naïve. All enrolled individuals were free from any evidence of infection.

Preparation of monocytes and neutrophils

Human monocytes and neutrophils were purified from the heparinized blood (20 U/ml of heparin) of healthy donors and GC patients (Heo *et al.*, 2006). Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll Hypaque-1077 gradient. Primary CD14⁺ monocytes were separated from PBMCs by negative selection with CD14⁺ (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Neutrophils were purified from adult peripheral blood by centrifugation on a Polymorphprep™ gradient as described previously (Heo *et al.*, 2006). After two washes with HBSS without Ca²⁺ and Mg²⁺, cells were resuspended in the appropriate medium. Isolated monocytes and neutrophils were more than 95% pure as analyzed by flow cytometry and Giemsa staining of cytopsin preparations (data not shown).

Flow cytometry

Monocytes (CD14⁺ cells) and neutrophils were incubated in 20% AB-serum in PBS at 4°C for 30 min. Subsequently, they were washed twice with FACS buffer (PBS containing 0.3% BSA and 0.1% NaN₃) and incubated with FITC-conjugated anti-HVEM-FITC mAbs or FITC-conjugated isotype control mAbs (Mouse IgG1) at 4°C for 30 min. Samples were analyzed by flow cytometry using a Becton Dickinson FACScan flow cytometer, and percentages of mHVEM⁺ cells were calculated with CELL-Quest software (BD Biosciences, San Jose CA).

Cytokine ELISA

Sera from patients with GC and healthy controls were harvested and frozen at -80°C. Cytokines were assayed with ELISA kits for IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 and TNF-α.

Monocyte culture and ELISA assay for soluble HVEM

Monocytes were purified from healthy controls and adjusted to 2×10^6 cells/ml in RPMI1640 medium containing 10% FBS. They were cultured at 37°C in the presence of various concentrations of rhLIGHT, rhIL-8, rhTNF-α, rhIL-2, PMA, ionomycin, LPS, PHA or A23187. After 24 h, cell-free supernatants were collected by centrifugation and frozen at -80°C.

To measure sHVEM in sera and culture supernatants, sandwich ELISAs were employed using two anti-HVEM mAbs (108 and 122) recognizing different epitopes (Heo *et al.*, 2007). A standard curve was constructed with HVEM-Fc as the antigen. ELISA plates were coated with 100 µg/well of mAb108 (1 µg/ml) and incubated overnight at 4°C. After incubation, plates were washed and blocked with PBS containing 4% BSA. Samples diluted in PBS containing 4% BSA were added, and plates were incubated overnight at 4°C. After washing, 100 µl/well of biotin-labeled mAb122 at 1 µg/ml in 4% BSA were added and incubation continued for 1 h at 37°C. After addition of streptavidin-HRP, plates were developed with TMB substrate solution (Endogen, Pierce Biotechnology, Rockford, CA), and absorbencies at 450 nm were determined. A standard curve constructed with HVEM-Fc (R&D system, Minneapolis, MN) indicated that the sensitivity limit was 16 pg/ml. HVEM concentrations in sera and supernatants were expressed as means ± SEM of quadruplicate samples.

Bactericidal activity

Staphylococcus aureus (ATCC 56389; American Type Culture Collection, Manassas, VA.) was grown in nutrient broth (Becton Dickinson, Sparks, MD.) and *Helicobacter pylori* (ATCC 26695) was grown in Brucella broth (Becton Dickinson). Aliquots were frozen at -80°C and thawed prior to use. Killing of bacteria was measured as previously described (Heo *et al.*, 2006). Briefly, overnight cultures of *S. aureus* (1×10^7 cells/ml) and *H. pylori* (1×10^7 cells/ml) were opsonized by incubation with 0.1% gelatin (w/v) and 10% (v/v) human AB serum in HBSS. One ml of HBSS containing 1×10^7 opsonized bacteria was added to 1×10^7 cells/ml of monocytes or neutrophils in 100 µl of HBSS and incubated at 37°C for 3 min with continuous rotation to promote phagocytosis. Non-ingested bacteria were discarded by differential centrifugation for 5 min at 1200 rpm, and cells containing ingested bacteria were cultured at 37°C for 10 min with slow rotation in the presence of rhLIGHT or heat inactivated rhLIGHT. Killing was stopped by spinning the cells on ice after addition of 1 ml distilled water containing 0.01% BSA. Cell clumps were disrupted by vigorous vortexing, and the number of viable bacteria was determined by plating 10-fold serial dilutions on agar plates. The % killing was calculated as follows: % killing = $1 - (\text{the number of viable bacteria at time } t / \text{the number of}$

viable bacteria at time 0)] \times 100.

Measurement of reactive oxygen species (ROS) by FACS

ROS generated by monocytes and neutrophils were assayed with the ROS-sensitive fluorescent dye, 2', 7'-dichlorofluorescein diacetate (DCF-DA). Cells were stained with 5 μ M DCF-DA for 15 min at 37°C in the dark. After incubation with DCF-DA, they were washed twice with PBS buffer and resuspended in FACS buffer. DCF-DA-stained monocytes were then treated with rhLIGHT for 45 min. After washing, they were analyzed by flow cytometry (FACS Calibur; BD Biosciences, San Jose CA), and numbers of DCF-DA positive cells were calculated and displayed in the form of histograms (Bass *et al.*, 1983).

Statistics

Differences between experimental groups were evaluated by Student's *t* test. Results are representative of at least three independent experiments from different blood donors. Data are presented as means \pm SEM.

Acknowledgements

We thank Dr. Kwon Byeong S for permission to use anti-HVEM antibodies. This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (BRL2009-0087350). The first three authors contributed equally to the work.

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