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Brief communication (Original)

Role of vascular endothelial growth factor receptor in the pro-proliferation activity of CD40-CD40L in AGS gastric cancer cells

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Background: CD40 is a type α-membrane protein of the tumor necrosis factor receptor super-family, and CD40-induced responses may mediate growth and angiogenesis in carcinoma cells.

Objectives: Define the effect of CD40 ligation on AGS gastric cancer cell line and the role of vascular endothelial growth factor/vascular endothelial growth factor receptor (VEGF/VEGFR) signals in this process.

Methods: We treated AGS cells with 1 μg/mL soluble CD40 ligand (sCD40L) with or without pre-incubation of either anti-VEGF mAb (MAB293) or VEGFR tyrosine kinase inhibitor (SU5416). We determined the growth effects by cell counts or [^{3}H]-thymidine incorporation assay and VEGF levels in cell-free supernatant using enzyme-linked immunosorbent assays.

Results: The engagement of CD40-induced AGS cells proliferation accompanied by a significant increase autocrine VEGF through PI3K activation ($p < 0.05$), and exogenous VEGF alone had no effect on spontaneous cell growth. SU5416 with a concentration of 8 μM lead to a dramatic decrease in cell survival induced by sCD40L ($p < 0.05$), whereas MAB293 did not have the similar effect ($p > 0.05$).

Conclusion: CD40-CD40L interaction promoted AGS cancer cell line proliferation through a VEGFR-dependent signal pathway in the presence of an internal autocrine loop.

Keywords: CD40**,** gastric cancer, sCD40L, VEGF, VEGFR

Gastric cancer (GC) is one of the most common cancers worldwide. Despite the efforts and advances in the diagnosis and treatment, GC remains the second leading cause of cancer mortality [1]. It was suggested that the tumor micro environment played a prominent role in clonal expansion of gastric cancer cells, which progressively accumulated *in vivo*, whereas it rapidly underwent apoptosis when cultures *in vitro* [2]. An increasing number of molecules had been found to associate with gastric cancer cells [3].

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CD40, a type membrane protein of the tumor necrosis factor receptor (TNFR) super-family, was expressed on a variety of normal cells such as B cells, macrophages, dendritic cells, and epithelial and endothelial cells. CD40 ligand (CD40L, also called CD154), a type integral membrane protein, was mainly expressed in activated T cells, B cells, and platelets. The interaction between CD40 and CD40L was crucial for both innate and adaptive immune responses [4, 5]. Previous studies showed that the activation of CD40 signal lead to B lymphocytes proliferation, germinal center formation, isotype switching and memory B cells induction [6]. Furthermore, the genetic mutations of CD40L could make the protein incapable of engagement with CD40 resulting in the defection of T cell function and cell-mediated immunity [7].

Moreover, CD40 was found on a wide range of tumor cells, including malignant hemopoietic cells like multiple myeloma (MM), lymphoma, and leukemia, and solid tumors like liver, bladder, breast, and gastric cancers [8]. Similarly, CD40-induced responses mediate growth and angiogenesis in carcinoma cells [9, 10]. Thus, CD40 expression might serve as a prognostic marker in lung cancer, which correlated with metastatic spread and poor prognosis [11]. Those results imply that the CD40 pathway is promisingly exploited for cancer therapy. Some investigators reported that AGS gastric cancer cell line had high-level expression of CD40 [12, 13]. In the present study, we investigated the effect of CD40 stimulated by sCD40L on AGS gastric cancer cell line and the mechanism, and demonstrated that VEGFR was essential for sCD40L-induced proliferation.

Materials and methods

Materials

AGS human gastric cancer cells were obtained from the American Type Culture Collection. Ham's F12 medium and fetal bovine serum (FBS) were from Invitrogen (Rockville, USA). The phosphoinositide 3-kinase (PI3K) inhibitor LY294002 and vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitor SU5416 were purchased from Calbiochem. Human vascular endothelial growth factor (VEGF) Sandwich enzyme-linked immuno-sorbent assays (ELISA) kit, anti-human VEGF neutralizing monoclonal antibody (mAb) (MAB293), recombinant human VEGF were all from R&D Systems (Minneapolis, USA). [^{3}H]-thymidine was from GE Healthcare Bio-Sciences (Piscataway, USA).

Cell line culture

AGS, a CD40 positive gastric cell line free of mycoplasma contamination, was maintained in F12 medium supplemented with 10% (*v/v*) FBS at 37^{o}C in a humidified 5% CO_2 atmosphere incubator (Jouan, Saint-Herblain, France). The culture media was changed 24 hours before each experiment, and AGS cell line was incubated with sCD40L for 24 hours or indicated time when cells confluence was 80%. The cell-free supernatants were collected, and the levels of VEGF were measured according to manufacture's guidelines.

[^{3}H]-thymidine incorporation assay ([^{3}H]-TdR)

The proliferation was measured by the assay of [^{3}H]-TdR as described previously [14]. Cells were inoculated in triplicate at 4x10^4 cells/well (96-well plate) (96-well plate) in 100 μL of F12 (10% FBS) with the indicated treatments. [3H]-thymidine (1 μCi) was added and incubated for the last 16 hours. Then, cells were counted in a liquid scintillation counter (Beckman-Coulter, Fullerton, USA) using the following:

$$SI = \frac{\text{Experimental group (cpm) - Backgroup (cpm)}}{\text{Control (cpm) - Backgroup (cpm)}}$$

Statistical analysis

The statistical analysis of the data was performed using SPSS 13.0 software, comparing the means between two groups using the Student's t-test.

Results

CD40 ligation obviously profited the growth of AGS cells

To assess the potential proliferation effect of CD40L in activation of CD40 signaling, we examined the expression of CD40 in AGS cells and treated the cells with the sCD40L, and determined the effect by cell counts. As shown in **Fig. 1**, the AGS expressed membrane-bound CD40 molecule are on the surface and CD40L have induced cell proliferation.

CD40 ligation stimulated VEGF secretion in AGS cells through PI3K activation

AGS cells co-cultured with 1 μg/mL sCD40L consistently secreted increasing VEGF secretion, which was first evident at 16 hours lasting up to 72 hour. The highest relative increase compared with control was at 24 hour and this time point was used in further examinations. Next, we pretreated AGS cells with PI3K specific inhibitor LY294002 to prevent activation of PI3K to investigate the mechanism of VEGF production mediated by sCD40L since PI3K is the major pathway activated by CD40 engagement. Through our initial experiments, we found that 20 μM of LY294002 did not show any toxicity on the AGS for experimental period (24 hours) using trypan blue assay. AGS cells were cultured for 24 hours in the presence of 1 μg/mL sCD40L alone or in pre-incubation with PI3K inhibitor LY294002 (20 μM) for one hour. Inhibition of PI3K signaling pathways induced a significant VEGF decrease (**Fig. 2**).

VEGFR was essential for the pro-proliferation effect of sCD40L on AGS cells

Because CD40L activated AGS cells growth accompanied by up-regulation of VEGF production, we supposed that VEGF or VGFR signals played an important role in this response. Therefore, AGS cells were cultured in the presence or absence of either anti-VEGF mAb (1 μg/mL MAB293, isotype IgG as control) or VEGFR inhibitor (8 μM SU5416, 0.2% DMSO diluent for the SU5416 as control) for one hour before the addition of sCD40L (1 μg/mL). The VEGFR inhibitor SU5416 with a concentration of 8 μM lead to a significant decrease in cell survival induced by sCD40L, whereas anti-VEGF mAb did not have the similar effect (**Fig. 3**). We also found that exogenous VEGF alone had no effect on spontaneous cell growth ($p > 0.05$).

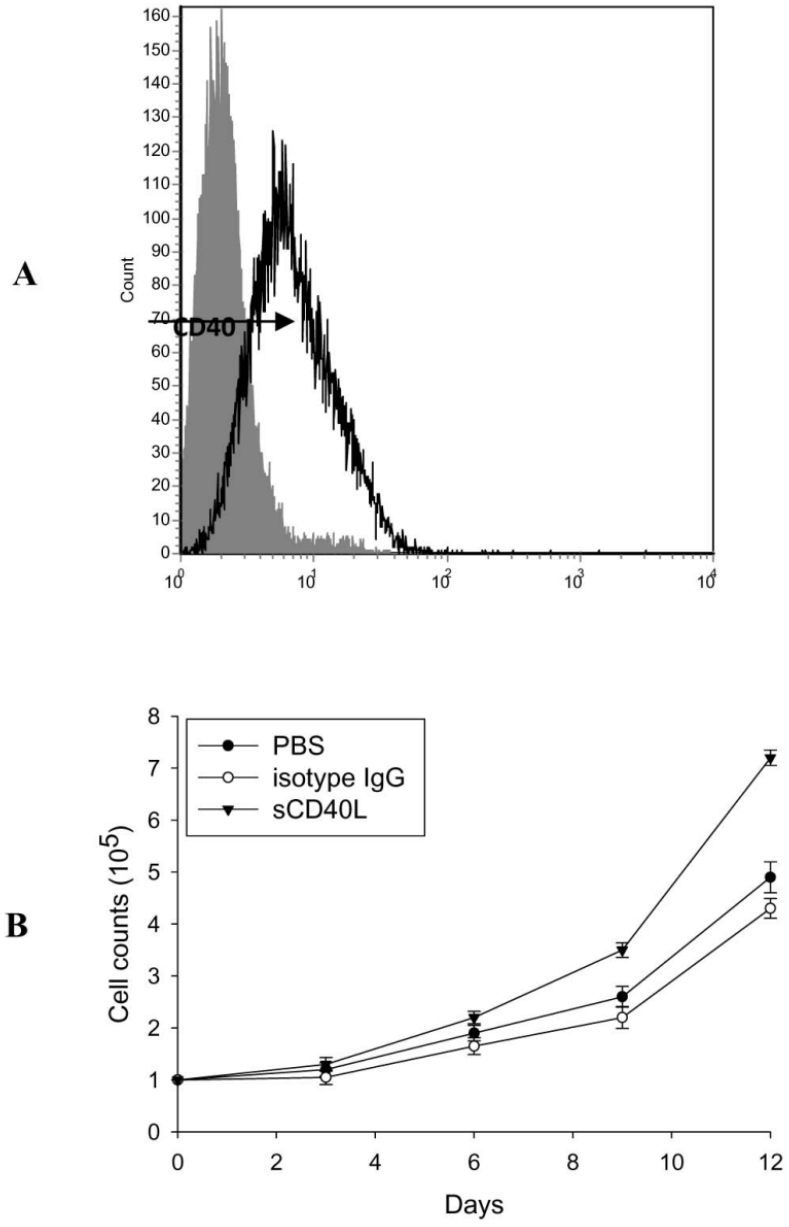

Fig. 1 The effect of sCD40L on the growth of AGS cells. **A**: Flow cytometric analysis of CD40 expression on AGS cells. The grey filled area indicates isotype control, the black line indicates anti-CD40 antibody. **B**: $1x10^5$ cells per well were cultured with 1 μg/mL sCD40L or PBS as control. The cells were counted under a microscope every three days. The results are presented as the mean of three separate experiments.

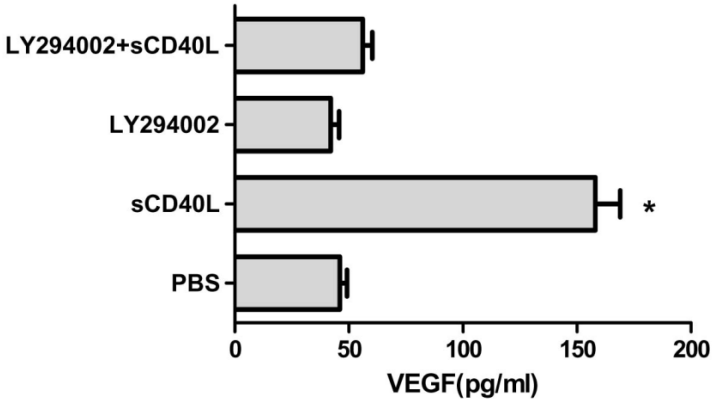

Fig. 2 The effects of CD40 activation on VEGF production in AGS cells. VEGF concentrations were measured by ELISA. Cells were cultured with PBS as negative control. The results are presented as the mean of three separate experiments. *p <0.05 compared with either PBS or LY294002+sCD40L groups.

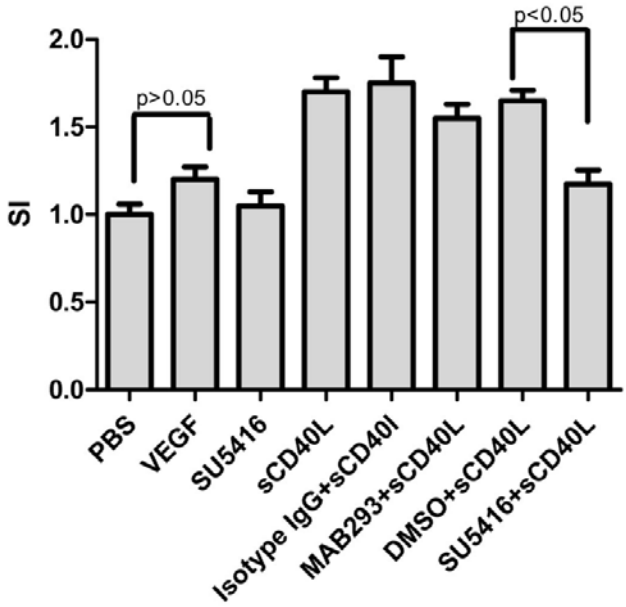

Fig. 3 The role of VEGF in pro-proliferation activity of sCD40L in AGS cells. Cell viability was measured using [3H]-TdR incorporation after four days culture. The results are presented as the mean of three separate experiments.

Discussion

CD40 lacked intrinsic catalytic kinase activity and transduced signals primarily through trimerization and recruitment of TNF receptor-associated factors (TRAFs) [15]. This resulted in downstream pathways including NF-κB activation to regulate the growth of cells. Here, we showed that CD40 played an important role in AGS gastric cancer cells and its ligation induced an apparent biological response of proliferation.

Numerous extracellular signals could activate PI3K pathway, which involved cell proliferation, survival, protein synthesis, and tumor growth. VEGF was a key mediator of tumor growth and angiogenesis induced by PI3K-induced signals for tumor growth and angiogenesis, PI3K/AKT signal regulated VEGF expression. In addition, it was critical for regulating VEGF expression and vice versa [16]. The activation of VEGF up-expression was triggered by PI3K, which was associated with CD40 after its ligation by sCD40L. It was reported that the ligation of CD40 on human endothelial cells induced Ras and phosphatidylinositol 3-kinase (PI3K) activation leading to the expression of and the promotion of angiogenesis

[17,18]. In the present study, the presence of PI3K inhibitor, LY294002, could inhibit PI3K activation, VEGF expression, and the pro-proliferation effect in AGS cells. This suggests that the increasing secrete of VEGF partially depended on PI3K activation. This was in accord with previous studies implicating PI3K in the up-regulating of VEGF production in various other cell types [19-22].

CD40-CD40L interactions and the associated expression of VEGF represented a mechanistic link between immunity and the development of angiogenesis [23]. However, we found that blocking autocrine VEGF with an anti-VEGF mAb did not reduce the growth promotion of sCD40L on AGS cells but blocking VEGFR signal pathway significantly did. Such divergent effects between external cytokine blockade and receptor-signaling inhibition had been reported on other cell types [10, 24]. We speculated that an internal autocrine loop that could not be inhibited by externally neutralizing antibody might exist in AGS cells. We also observed whether the presence of VEGF alone brought out the same effect as sCD40L. The result showed that exogenous VEGF could not stimulate AGS cells proliferation. This suggested that the pro-proliferation effect of VEGF secreted in response to the engagement of CD40 required both VEGFR and CD40L signals simultaneously.

Conclusion

Collectively, CD40-CD40L could induce AGS gastric cancer cells proliferation through a VEGFR-dependent mechanism with the presence of a VEGF autocrine function specifically linked to the regulation of cells survival.

Acknowledgments

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The authors have no conflict of interest to declare.

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