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Full Length Research Paper

Adenovirus vectors can induce activation of endothelial cells: CD40-CD40L interactions partly participate in the endothelial cells activation induced by adenovirus vectors in an NF-kappaB-dependent manner

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Replication-defective adenovirus vector without both E1 and E3 is one of the most popular tools in transgenic therapies. However, more attention should be paid to adenovirus vectors mediated-gene modified study on endothelial cells (ECs). To verify the possible danger in that process, we explored the effect of adenovirus on ECs in this study. By using western blot analysis, we showed that the level of both CD40 and CD40L on human umbilical vein endothelial cells (HUVECs) were upregraduated by adenovirus vector infection at 100 multiplicity of infection (MOI). The activation of ECs induced by adenovirus vector infection at MOI 100 can be partly inhibited by a blockade of CD40/CD40L interactions by using the recombinant adenovirus Ad-sCD40LIg or an anti-CD40L monoclonal antibody (mAb) *in vitro*. On ECs, blockade of CD40/CD40L decreased the expression of IL (interleukin)-6, IL-8 and intercellular adhesion molecule (ICAM) in adenovirus vector-induced cells. In electrophoretic mobility shift assay (EMSA), both Ad-sCD40LIg and anti-CD40L mAb can attenuate the activity of NF-kappaB (NF-κB) pathway contributing to the activation of ECs, which indicated that CD40-CD40L interactions played significant role in the activation of ECs induced by adenovirus vectors via an NF-κB pathway. Our study provide evidences for a supplementary mechanism of the ECs activation induced by adenovirus vector infection and suggests that CD40-CD40L interactions partly participate in the ECs activation induced by adenovirus vectors in an NF-κB-dependent manner.

Key words: Adenovirus vector, CD40, CD40L, endothelial cells, NF-kappaB.

INTRODUCTION

Replication-defective adenovirus vector without both E1 and E3 is one of the most popular tools in transgenic therapies for its advantages, for instance they can carry long exogenous DNA fragments, infect variety of dividing and nondividing cells at higher infectious rate and there is less risk of inducing alteration of the host DNA for the infection cannot interfere with the host genome (Kanaya et al., 2003). However, some problems must be tackled before the vector is applicated for patients. Adenovirus vectors, regardless of containing an exogenous or not, can excite an inflammatory reaction followed by upregulation of the inflammatory genes (Yei et al., 1994; Schaack et al., 2011). Adenovirus vectors can promote the acute inflammation *in vivo* by inducing chemokines including the C-C chemokine RANTES and IP-10 through

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Abbreviations: **ECs**, Endothelial cells; **HUVECs**, human umbilical vein endothelial cells; **MOI**, multiplicity of infection; **mAb**, monoclonal antibody; **NF-κB**, NF-kappaB.

capsid dependent activation of NF-κB (Borgland et al., 2000; Bowen et al., 2002). Similarly, replication-deficient adenoviral vectors can induce the pro-inflammatoin via the NF-kB pathway in respiratory cells (Melotti et al., 2001).

Vascular endothelial cells (ECs) represent the natural barrier between the blood and surrounding nonvascular tissue. Besides the barrier function, ECs also plays vital roles during inflammation process by regulating leukocyte recruitment via the expression of inflammatory genes such as E-selectin, VCAM, ICAM, IL-6 and IL-8 (Ridley et al., 1997; Saccani et al., 2002; Gustin et al., 2004; Viemann et al., 2004; Kuldo et al., 2005) and actively participate in the processes of angiogenesis, vascular remodeling, and tumorigenesis (Folkman, 2003; Armulik et al., 2005; Tammela et al., 2005). As we know, resting ECs can be mainly activated by TNF-α and IL-1β inducing to upregulate the expression level of the cytokine and cell adhesion moleculars (CAMs) via the NF-κB pathway (Modur et al., 1996; Dechanet et al., 1997; Stehlik et al., 1998; Gawaz et al., 2002; Zhou et al., 2007). Study on gene modification of ECs or other cells lines mediated by replication-defective adenovirus vectors has been broadly applied in many aspects including protein expression and RNA silence (Lin et al., 2004; Kim et al., 2007; Ritchie et al., 2007). A study showed that adenovirus vectors can stimulate maturation of dendritic cells, on which the CD40 protein expression was obviously upregulated (Morelli et al., 2000). However, limited data are available on whether replication-deficient adenoviral vectors can activate the ECs via the NF-κB pathway.

CD40/CD40L (the ligand of the CD40, also known as CD154) pathway plays critical roles in inflammation and immune regulation, including the activation of ECs (Henn et al., 1998). Some studies show that CD40 was expressed on ECs (Grewal and Flavell, 1998; van Kooten and Banchereau, 2000; Bergmann and Pandolfi, 2006). CD40L is also shown to be expressed on ECs, besides on T cells, B cells, monocytes, macrophages and dendritic cells (Mach et al., 1997; Arciniegas et al., 2003; Cognasse et al., 2007). However, only one literature (Geraldes et al., 2006) reported that unstimulated ECs coexpressed both CD40 and CD40L protein in a low basal level. CD40L can induce ECs activation to generate signals for the recruitment and extravasation of leukocytes at the site of injury by secreting chemokines and expressing CAMs (Dechanet et al., 1997; Henn et al., 1998). Base on a study that ligation of CD40 ligand (CD40L) delivers signals to the CD40L bearing cells themselves (van Kooten and Banchereau, 1997), we speculated that adenovirus vectors could upregulate the expression of the CD40 and (or) CD40L to participate in the activation of ECs. Therefore, in this study, we will investigate the effect of adenovirus vectors on the expression of the two proteins described previously and whether or how adenovirus vectors affect the activation status of ECs.

MATERIALS AND METHODS

Cell culture

Our study was approved by the Institutional Ethics Committee and the original donors of the cells gave their consent to participate in this research. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords as described by Lin et al. (2005). Briefly, the umbilical vein was filled with 20 ml of 0.1% type Ⅱ collagenase (Sigma, St. Louis, MO) dissolved in phosphate-buffered saline (PBS) and incubated for 15 min at 37℃. The collagenase solution was drained from the cord and collected. The cells in the pooled solutions were recovered by centrifugation at 1000 rpm for 5 min and transferred to dishes under standard conditions in medium M-199 (Sigma) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), heparin (15 U/ml) (Biochem pharmaceutical plant, nanjing, China), recombinant human endothelial cell growth factor (rHuVEGF, 20 μg/ml) (GenScript, Nanjing, China), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). The isolated cells were identified as endothelial cells by phasecontrast microscopy and immunocytochemistry detection of factor Ⅷantigen (Promega Life Sciences, Madison, WI).

Western blot

Western blot analysis was performed as described previously by Li et al. (2006). Briefly, ECs were infected by control adenovirus Ad-CMV (Stratagene, CA, USA) at 100 MOI (multiplicity of infection). Twenty microgram protein extracts from ECs with or without treatment by adenovirus vector were fractionated by 8% SDS-PAGE and transferred to cellulose nitrate membrane. After blocking, the membranes were incubated at 4℃ overnight in Tris-buffered saline (TBS; 50 mmol/l Tris-HCl, 150 mmol/l NaCl) containing a 1:1000 dilution of rabbit-anti-human CD40L or CD40 antibody (Santa Cruz, CA, USA) and then incubated for 1 h at room temperature in TBS containing a 1:2000 anti-rabbit IgG antibody conjugated horseradish peroxidase (Santa Cruz). Immunoreactive bands were visualized by incubation with LumiGLO (Cell Signaling Tech, MA, USA) and exposure to light-sensitive film.

EMSA (electrophoretic mobility shift assay)

To analyze the role of CD40/CD40L pathway in the activation of endothelial cells induced by adenovirus vector, we choose two ways to block the CD40/CD40L pathway: Anti-CD40L monoclonal antibody (mAb) (kindly provided by Dr. Xuan LIANG, Medical School, Xi'an Jiaotong University, China), Ad-CD40Llg recombinated in our previous study and the blockade effect of Ad-CD40Llg on CD40/CD40L pathway had been confirmed (Li et al., 2006). ECs were plated out at a density of 5×10^5 cells/well on a 6-well plate to adhere overnight and were treated as follows: Group 1, resting ECs without treatment. Group 2, ECs were infected by Ad-CMV at 100 MOI. Group 3, ECs were infected by Ad-sCD40Llg at 100 MOI, Group 4, ECs were infected by Ad-CMV at 100 MOI and the CD40/CD40Llg pathway was blocked by Anti-CD40L mAb (1 microgram/ml). NF-κB activity was analyzed using EMSA. Nuclear extracts were prepared following the method described by Schreiber et al. (1989). EMSA was performed as described previously by Kiemer et al. (2002). Consensus binding sequence for NF-κB is 5'-AGT TGA GGG GAC TTT CCC AGG C-3', DNA probes were labeled with [γ-32P]ATP using T4 polynucleotide kinase (Boehringer Mannheim, Mannhein, Germany) and purified using pharmacia NICK columns (Promega, Life Sciences). Briefly, a portion (1 mg) of each sample of nuclear protein was mixed with the incubation buffer, and the mixture was preincubated at 4℃ for 15

min. The labeled oligonucleotide was added and the mixture was incubated at room temperature for 20 min. The final mixture were loaded on to a 6% nondenaturing polyacrylamide gel and resolved by electrophoresis.

Flow cytometry

The level of ICAM-1 expression in ECs was measured by flow cytometry. ECs with or without treatment as described earlier were detached by 0.25% trypsin and 0.01% EDTA (Sigma) for 1 min at 37℃ and washed with cold phosphate-buffered saline (PBS). Then, approximately 1×10^6 suspended cells in 500 mcroliter PBS were incubated with 10 µl phycoerythrin-conjugated anti-human ICAM-1 monoclonal antibody (Becton Dickinson, San Jose, CA, USA) for 30 min at 4℃ in darkness. A mouse isotype (IgG2) antibody (Becton Dickinson) was used as control. The cells were washed three times with cold PBS, fixed with 1% paraformaldehyde (Sigma) and analyzed on a flow cytometer (FACSCalibur, Becton Dickinson). Ten thousands of ECs were evaluated for each sample. The data were analyzed by cell quest software version 2.0 for MACOS (Becton Dickinson).

Cytokines and sICAM-1(soluble form of the ICAM-1) measurement

ECs were treated as described earlier. At 24 h after adenovirus infection, 100 µL supernatant was collected and microfuged briefly to pellet cell debris. ELISA was carried out for IL-6, IL-8 and sICAM-1 using a kit purchased from Biosource International (Camarillo, California, USA) according to the manufacturer's instructions. Experiments were performed in triplicate for every culture condition.

Statistic analysis

Experiments were performed thrice and data are presented as mean±SD. The data was analyzed via ANOVA by using SPSS for Windows, version 12.0 (SPSS Inc, Chicago, IL). A p-value less than 0.05 were considered statistically significant.

RESULTS

Protein levels of CD40 and CD40L on ECs were upregulated by adenovirus vectors

To determine the effect of Ad-CMV on the protein level of CD40 and CD40L on ECs, western blotting analysis was performed as described in materials and methods section. We observed a minimal basal expression level of both proteins in resting ECs. A concentration-dependent stimulation of ECs by adenovirus vectors was performed and we observed that maximal effect on CD40 and CD40L protein expression was achieved after 24 h of infection with 100 MOI of adenovirus vectors (data not shown). The 24 h infection with adenovirus vectors (MOI=100) upregulated the protein expression of CD40L and CD40 by 44.02 and 115.77%, respectively (Figure 1).

Blockade of CD40/CD40L pathway inhibits partly ECs activation induced by adenovirus via attenuating NF-κB activatity

To analyze the role of CD40/CD40L pathway in the activation of endothelial cells induced by adenovirus vector, we choose two methods to block the CD40/CD40L pathway: Anti-CD40L mAb and Ad-CD40LIg. ECs were treated as previously described. The super shift assay and excess cold probe competition were not performed in the present study because the specificity of the same probe binding to NF-κB protein had been confirmed in previous study (Palmer et al., 2005). In the EMSA, unstimulated ECs extract showed small amount of DNA-protein complexes, which were increased after Ad-CMV stimulation (lane 5). Compared to Ad-CMV stimulation group, DNA-protein complexes were decreased after the Ad-sCD40LIg or Anti-CD40L mAb treatment (lane 4 and 5, respectively), which were more obvious in the Anti-CD40L mAb treated group.

IL-6, IL-8, ICAM-1 or sICAM expression increase after adenovirus vector infection

To investigate the effect of adenovirus vectors on the cytokine and ICAM expression on ECs, ECs were treated as described in materials and methods section. The expression level of IL-6 and IL-8 was detected by ELISA. The results show that the expression level of IL-8 in cell supernatant was upregulated by adenovirus vector infection compared to ECs unstimulated or infected by adenovirus vectors plus the blockade of the CD40/CD40L pathway ($p<0.001$, group1, group2 ,group3 vs. group4). And the IL-6 level also increased in the adenovirus vectors transfected group ($p<0.001$, group1, group2 vs. group4; $p=0.011$, group3 vs. group4). Compared to the resting ECs, the group2 had no difference in IL-6 expression ($p=0.061$) though IL-6 level increased in the Ad-CD40LIg treated group ($p=0.010$, group3 vs. group1) (Figure 3). Then FCM was performed to determine the protein expression of ICAM-1. The result shows that the ICAM expression population was increased to about 50% ($p<0.05$, group1, group2, group3 vs. group4) , though the population of ICAM expression cells was about 17 and 24% in both groups in which the CD40/CD40L pathway had been blocked (Figure 4A). In addition, for consideration of the fact that the sICAM-1 also participates in the inflammation induced by ECs activation, ELISA also was performed to observe the sICAM-1 level in cell supernatant. The results show that sICAM-1 level in adenovirus vector transfected ECs supernatant was increased compared to the other three groups ($p<0.001$, $p=0.020$ and $p=0.024$, respectively, Figure 4B).

DISCUSSION

Adenovirus vectors can upregulate the expression of CD40 and CD40L on ECs

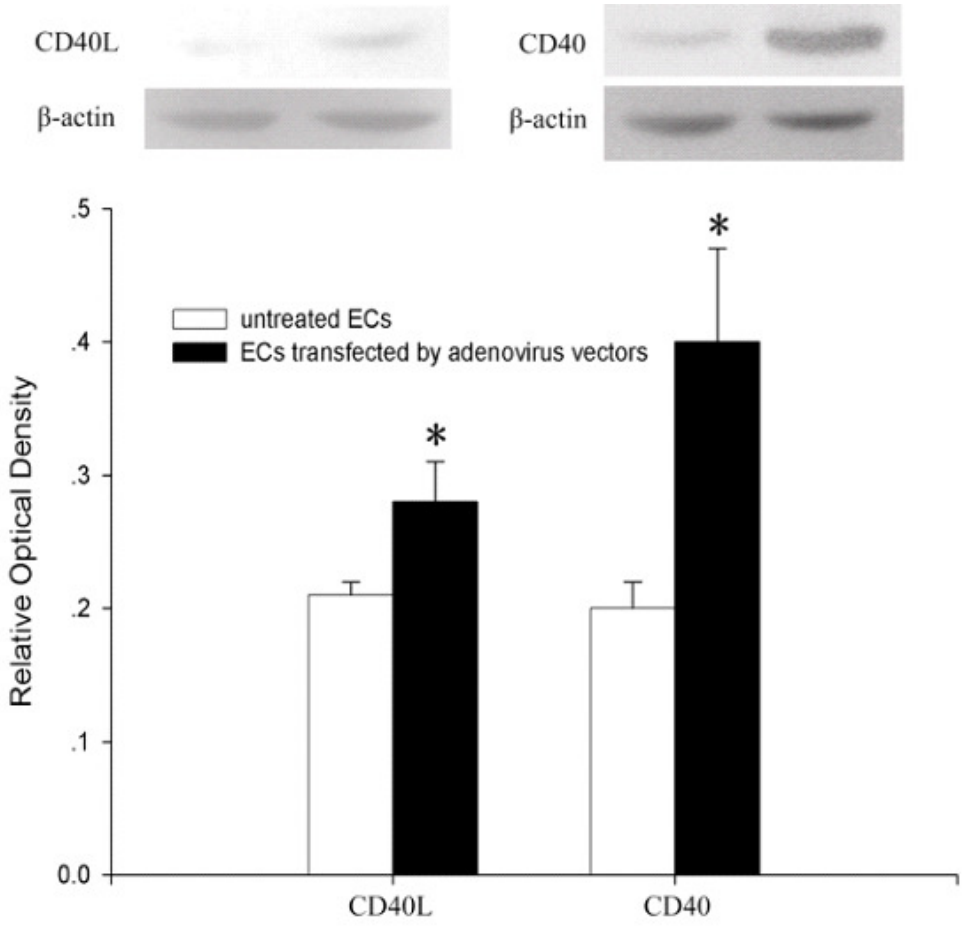

Figure 1. The expression of CD40L and CD40 was upregulated by adenovirus infection. Western blot analysis was used to determine the expression of CD40L and CD40 in treated or untreated group, (The picture represent one of three independent experiments). The results of western blot analysis were quantitated. The relative optical density (OD) was calculated as OD of each protein band compared to that of β-actin. The open column represented untreated EC group and closed column represented adenovirus vectors transfected group. *p<0.05 vs. untreated ECs.

(Geraldes et al., 2006; Reul et al., 1997), while the expression of CD40 is also been observed on B cells, T cells and unstimulated ECs (Yellin et al., 1995; Mach et al., 1997; Arciniegas et al., 2003; Geraldes et al., 2006; Cognasse et al., 2007). Cultured endothelial cells were found to express little constitutive CD40L which was markedly increased after 24 h of treatment with TNF-α, IL-1α, IL-4, or IFN-γ (Reul et al., 1997). Another study showed that adenovirus vectors can unregulat the CD40 protein expression on dendritic cells (Morelli et al., 2000). C-reactive protein (CRP) can induce CD40 expression in HUVECs partly via activation of NF-kappaB (Lin et al., 2005). However, data about the effect of adenovirus vectors on both proteins expression on ECs are limited. Therefore, in our study, we first and foremost investigated the expression of CD40 and CD40L on ECs treated or untreated by adenovirus vector infection. Consistent with other study (Geraldes et al., 2006); we observed a minimal basal expression level of both proteins by western blot analysis. In addition, we reported that the expression profile of CD40L and CD40 proteins was obviously upregulated by adenovirus vector infection on ECs. Patients suffering from peripheral arterial occlusive disease presented high CD40L and showed significantly higher soluble VCAM-1 and soluble P- and E-selectin (Tsakiris et al., 2000), which implied CD40-CD40L significant role in proinflammatory associated diseased. As we know, CD40-CD40L interaction has been emphasized on by investigator for its important role in many pathogenesis, such as artherosclerosis (Phipps,

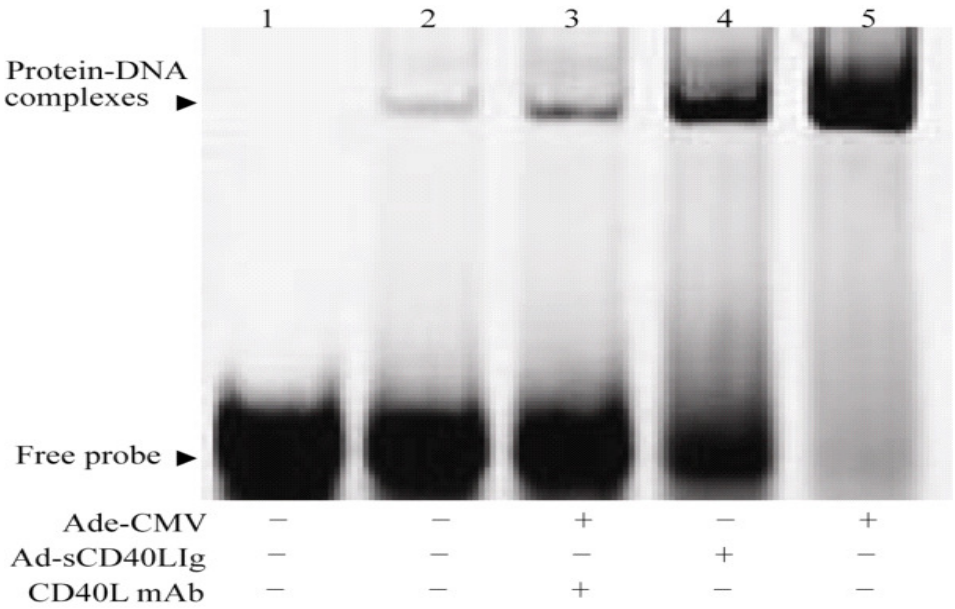

Figure 2. Adenovirus vectors activate ECs via NF-κB pathway. Group 1, resting ECs without treatment as negative control (lane 2). Group 2, ECs were infected by Ad-CMV at 100 MOI (lane 3). Group 3, ECs were infected by Ad-sCD40LIg at 100 MOI (lane 4). Group4, ECs were infected by Ad-CMV at 100 MOI and the CD40/CD40LIg pathway was blocked by Anti-CD40L mAb (1 µg/ml) (lane 5).Lane 1 showed free probe.

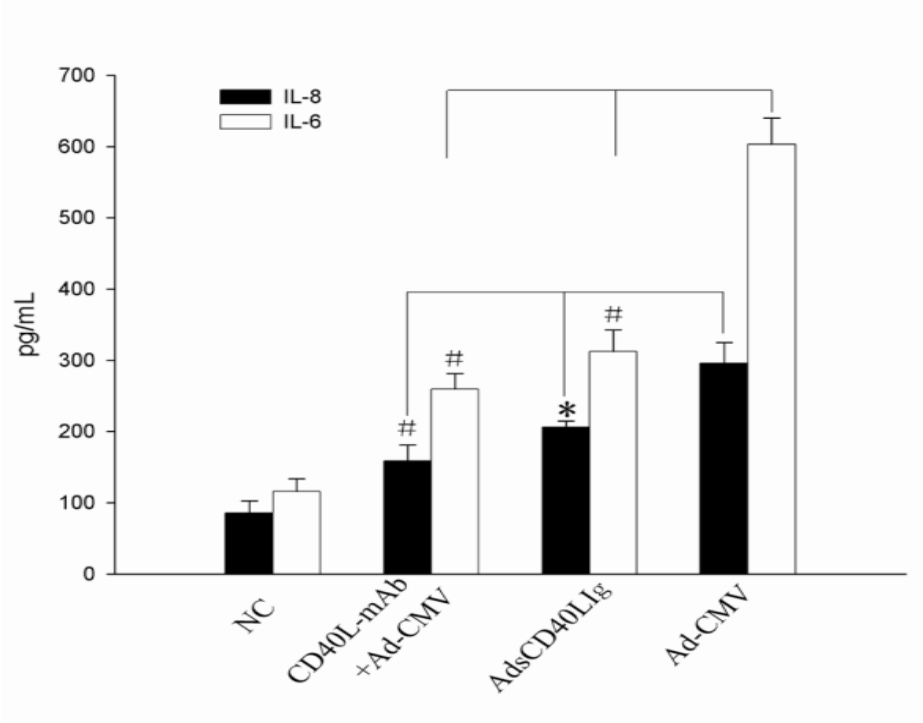

Figure 3. IL-8 and IL-6 expression level upregulated by adenovirus vector infection were analysised by using ELISA. The open column represented IL-6 level in cell supernatant in different treated groups, p=0.061(group2 vs. group1), p=0.010(group3 vs. group1), p<0.001. (group4 vs. group1), p<0.001 (group2 vs. group4), p=0.011(group3 vs. group4). The closed column represented IL-8 level in cell supernatant in different treated groups, p<0.001 (group2, group3, group4 vs. group1), p<0.001 (group2, group3 vs. group4). *p<0.05,

#p<0.001.

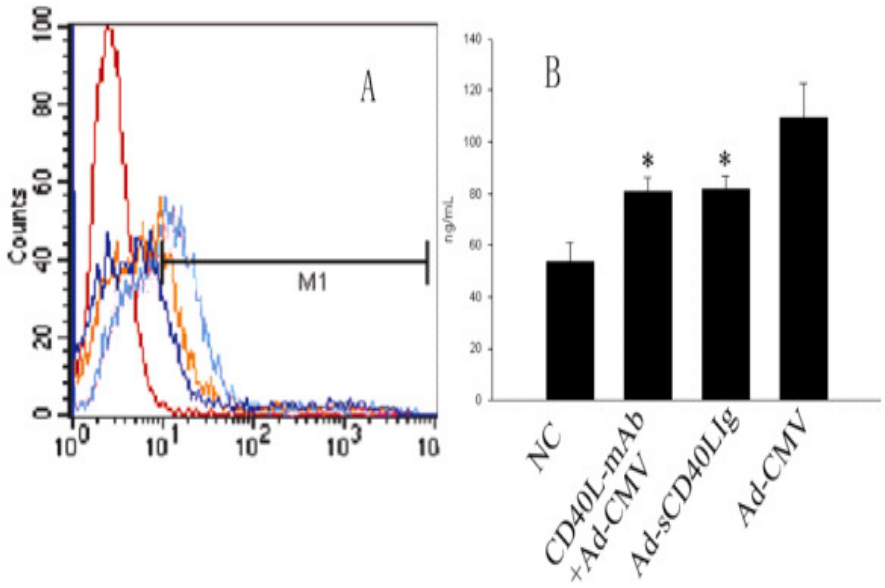

Figure 4. ICAM and sICAM expression level upregulated by adenovirus vector infection were analysised by using FCM and ELISA. A, The ICAM expression population was determined by FCM: NC (—). Ad-CMV transfected (MOI=100) plus CD40 mAb-treated group (—), 16.95 %. Ad-sCD40LIg-treated group (—), 24.10%. Ad-CMV transfected group (—), 50.33%. The picture is one present of three independent experiments. B, The column represented sICAM level in cell supernatant in different treated groups, (p<0.001, p=0.020 and p=0.024, respectively. group1, group2 and group3 vs. group4), *p<0.05.

al., 1997; Berner et al., 2000), graft rejection (Zheng et al., 1998; Gaweco et al., 1999), unstable angina (Aukrust et al., 1999) and other inflammation associated diseases especially. Though, adenovirus vector is convenient alternative for gene modified, adverse side effects should be paid attention to, for instant, the upregulation of CD40L and CD40 expression may contribute to proinflammatory reaction.

ECs can be activated by adenovirus vector infection via NF-kappaB pathway

The fact that ECs can be activated by both TNF-alpha- and IL-1beta-induced had been established in many studies. In addition, C-reactive protein (CRP) and lipopolysaccharide (LPS) also induced the activation of ECs (Wrighton et al., 1996; Lin et al., 2005). In those investigations, NF-kappaB pathway played a major role in the ECs activation which had been confirmed. Both the NF-kB and MAP kinase pathways are reported to be activated by adenovirus infection of respiratory epithelium, hepatocytes and vascular smooth muscle cells (Clesham et al., 1998; Melotti et al., 2001; Tamanini et al., 2003). Palmer et al. (2005) showed that adenovirus vectors activated the PI3-kinase/AKT pathway, ERK/MAPK pathway and NF-kB pathway in a number of human carcinoma cell lines. Adenovirus vector infection resulted in the phosphorylation of IkBα and the consequent nuclear relocalization of the transcription factor NF-kB. In their study, an obvious activation of NF-Kb induced by adenovirus vector infection had been observed, and it was demonstrated that NF-kB activation mediated adenovirus-induced inflammatory effects, which were exemplified by an increased secretion of the proinflammatory cytokine IL-6 and COX-2. However, to date, there are no reports regarding whether adenovirus vectors can activate ECs. In our study, we perform ELISA and FCM to analyze IL-6, IL-8 and ICAM-1 (or sICAM) level in ECs transfected by adenovirus vectors. Compared to resting ECs, the proinflammatory cytokine IL-6, IL-8 and ICAM-1(or sICAM) levels significantly increased in stimulated ECs. Consistent with our results, Wrighton et al. (1996) had reported that adenovirus infection had an effect on ECs: IL-lα and IL-6 mRNA levels were significantly enhanced in control virus-infected as compared to noninfected cells; In addition, infection with the control adenovirus slightly stimulated von Willebrand factor (vWF) secretion. Then, EMSA was performed to evaluate whether the NF-kappa B pathway can be involve in ECs activation. In this study, NF-kappaB-DNA complexes increased in ECs infected by

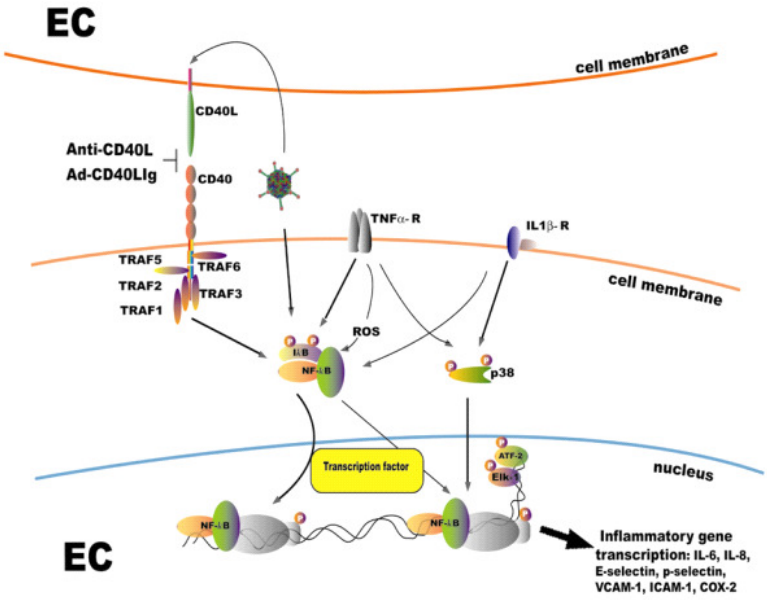

Figure 5. Schematic representation of signal transduction in endothelial cells. Simplified scheme of TNFα- , IL-1β- and adenovirus vector-induced signal transduction in endothelial cells and consequent inflammatory gene expression is shown. Triggering of TNF-α and IL-1β receptors (TNF-α-R and IL-1β-R) initiates events leading to NF-κB and p38 MAPK activation. p38 MAPK-based histone phosphorylation possibly prepares E-selectin, IL-6, IL-8, and COX-2 promoters for facilitated NF-κB binding, leading to the enhanced gene expression.

vectors can activate ECs via NF-kappaB activation.

CD40-CD40L interactions partly participate in the activation of endothelial cells induced by adenovirus vector in an NF-kappaB-dependent manner

CD40L expressed on platelets and neutrophil cells interacting with CD40 on ECs can induce ECs activation *in vitro*, which in turn leads to increase expression of leukocyte adhesion molecules, including E-selectin, vascular cell adhesion molecule 1, and intercellular adhesion molecule 1, and expression of cytokines including IL-6, IL-8, and cyclooxygenase (COX)-2 (Yellin et al., 1995; Karmann et al., 1995; Hollenbaugh et al., 1995; Karmann et al., 1996; Miller et al., 1998; Thienel et al., 1999; Tsakiris et al., 2000; Kuldo et al., 2005). The inducing of ECs activation by CD40L-CD40 interactions is thought to be mediated by the NF-kappaB pathway and the kinetics of CD40L-, interleukin 1-, or tumor necrosis factor alpha-induced ICAM, endothelial leukocyte adhesion molecule-1 (ELAM-1), and VCAM-1 up-regulation on ECs are similar (Yellin et al., 1995). A study found that blood dendritic cells (DCs) expressed a functional CD40L and stimulation through CD40 upregulated CD40L mRNA and protein expression in DCs, B cells, and B cell lines, in which the induction of CD40L expression via CD40 requires protein tyrosine kinase activity (Pinchuk et al., 1996). Another study reported that CD40L increased expression of its receptor CD40 in human coronary artery endothelial cells which may be mediated by oxidative stress and ERK1/2 activation (Chai et al., 2006). Another intriguing result addressed that ligation of CD40 ligand (CD40L) delivers signals to the CD40L bearing cells themselves (van Kooten and Banchereau, 1997). Therefore, CD40L and CD40 upregulate the protein expression in the other side and upregulation of CD40L or CD40 expression may increase the chance of inducing of ECs activation by CD40L-CD40 interactions. In this study, the expression of CD40 and CD40L on ECs was obviously upregulated by adenovirus vector infection. The upregulation of IL-6, IL-8 and ICAM-1 or sICAM expression can be blocked by anti-CD40L mAb or Ad-CD40LIg partly which made us believe that EC-EC interactions may exist though, direct cell-cell or indirect supernatant-cell CD40-CD40L contact way is unclear. In EMSA, we demonstrate that CD40-CD40L interactions partly stimulate ECs activation in an NF-kappaB-dependent manner. Therefore, besides PI3-kinase/AKT, p38/ERK/MAPK and NF-kB pathways, CD40/CD40L interactions partly at least participate in the activation of endothelial cells induced by adenovirus

vectors (Figure 5). Though, we focused on MOI=100 in this study, the effect of the lower or higher MOI

adenovirus vectors on ECs would be addressed in future study. As we know, *in vitro* or *in vivo*, inflammatory reaction would be excited after ECs is activated, which may result in unwanted toxicity and may therefore limit the systemic administration of adenovirus vectors. Therefore, adverse effect must be considered when ECs are modified by exogenous genes mediated by adenovirus vectors.

Conclusions

In conclusion, in this study, we demonstrate that adenovirus vector infection can upregulate the expression of CD40 and CD40L on ECs and can induce EC activation. CD40-CD40L interactions on ECs themselves at least partly participate in ECs activation with induction by adenovirus vector infection in an NF-kappaB-dependent manner. Therefore, attention should be given to adenovirus vectors mediated-gene modified study on ECs, although, this findings not be may appropriate for all the situations, such as lower or higher MOI adenovirus vector infection.

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