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Establishment of a T-Ag and NF-κB Binding Elements Gene Co-Transfected Stable HUVECs Cell Line

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Abstract To set up a *T-Ag* gene transfected stable human umbilical venous endothelial cells (HUVECs) cell line and a T-Ag, nuclear transcriptional factor kappa B (NF-κB) binding elements linked with luciferase reporter gene co-transfected stable HUVECs cell line. Cultured HUVECs were transfected with pCI-neo-T-Ag and pRSV-luc-3XκB by lipofectin. The G418 selected monoclones were subcultured. The expression of marker protein, vWF and the characteristic of uptake of lipids were compared by Western blotting and immunocytochemistry in non-transfected and transfected HUVECs. The reporter gene assay was done in the presence of TNF-α. A *T-Ag* gene transfected stable HUVECs cell line and a T-Ag and NF-κB binding elements linked with luciferase reporter gene co-transfected stable HUVECs cell lines were set up. The expression of vWF of these cell lines was similar with those in non-transformed HUVECs. The function of uptaking of lipids was preserved as well in transfected cell lines. Furthermore, TNF-α, a typical cytokine increasing the activity of NF-κB was used to treat the transfected cells O/N. The higher luciferase reporter gene activity was seen. A pCI-neo-T-Ag and pRSV-luc-3X κB co-transfected stable HUVECs cell line might be used to check reporter gene activity directly. It might be a useful tool to screen drugs acting on transcription level. J. Cell. Biochem. 89: 1285–1290, 2003.

Key words: cell line; co-transfection; T-Ag; NF-κB; reporter gene activity assay

Endothelial cells (EC) currently used as in vitro model systems for various physiological and pathological processes, especially in angiogenesis research. Primary EC have a limited lifespan and display characteristics that differ from batch to batch due to their multi-donor origin. The limited number of EC lines available and the difficulties in obtaining primary cultures of cells that maintained the structural and biochemical characteristics of endothelium has impeded many areas of research. To meet the needs of research several groups have established EC lines in recent years. Bouis et al. [2001] gave an overview of the currently available vascular EC lines in his review article. Usually, EC lines were obtained by addition of viral oncogenes [Michael et al., 1976; Fickling et al., 1992].

Nuclear transcriptional factor kappa B (NF-κB) was a common target to many upstream molecules. We had known that a lot of drugs acting on intracellular signaling pathways, such as PI3K-Akt survival pathway, promoted transcription and synthesis of proteins through activation of NF-κB. Once it was activated, the downstream gene expression occurred. Still there are some drugs, as well as some new compounds that we do not know if they acted on transcriptional level and promoted expression of downstream genes. To study the transient gene expression in mammalian cell culture lines, the technique of transfection with a reporter gene was commonly used. The plasmid containing reporter gene was transfected into cells using a liposome-mediated procedure. The most popular gene for these studies of transient gene expression in the past was the chloramphenicol acetyltransferase (*CAT*) gene. Recent advances in bioluminescent chemistries have provided the method of luciferase reporter gene assay, a simple, fast,

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non-isotopic, and highly sensitive alternative to the popular radioactive CAT. However, the transfection efficiency was lower and varied in cultured primary cells, such as human umbilical venous endothelial cells (HUVECs). So that co-transfection with a second reporter gene is often performed to normalize transfection efficiency between assays. Thus made the experiment more tanglesome, expensive, and time consuming.

The present study described a new, stable cell line of HUVECs co-transfected by two plasmids. One was a recombinant plasmid containing the SV-40 large T antigen gene and neomycin resistance gene. The other was a plasmid containing three NF-κB binding elements gene and a luciferase reporter gene. The former plasmid provided HUVECs an immobilized characteristic. The later one expressed NF-κB binding elements gene. Once activated by upstream molecules, NF-κB bound with its binding sites and promoted expression of luciferase protein. NF-κB activity could be estimated by measuring luminescent of luciferase protein. Stable and monoclonal transfected HUVECs cell lines were obtained through G418 selection. This selected stable HUVECs cell line would apply a useful platform to screen the drugs acting on transcriptional level by simply detecting luciferase activity directly without co-transfection with the second reporter gene plasmid in every experiment.

MATERIALS AND METHODS

Cell Culture

HUVECs were purchased from Clonetics (Walkersville, MD) and were cultured in endothelial growth medium (EGM) containing 1% penicillin– streptomycin in a humidified incubator with 5.0% CO_2 in air at 37°C. Cells were passaged by standard trypsinization and passages 2–3 were used in the experiments.

Plasmids Replication and Purification

The plasmid pCI-A58 containing the SV-40 large T antigen gene and neomycin resistance gene and the plasmid pRSV-luc containing three NF-κB binding elements gene and a luciferase reporter gene were grown in *E. coli*, respectively. The DNA was isolated with Qiagen columns. Eluted DNA was ethanol-precipitated, resuspended in sterile TE buffer (10 mM Tris-HCl, 1 mM EGTA, pH 8.0), and quantified by measurement of absorbance at 260 nm.

Transfection

DNA transfer into HUVECs was performed using a liposome-mediated procedure. Briefly, HUVECs were plated in p100-mm plastic culture plates at 50–60% confluent. Monolayers were allowed to attach for O/N in EGM. Cells were washed by Opti MEM (Gibco, Tulsa, OK) just before transfection. For pCI-A58-T-Ag transfected cells, diluted 6 μg DNA and 50 μg lipofectin (Invitrogen, Carlsbad, CA) in 500 μl Opti MEM, respectively, mixed well and allowed them to stand at room temperature for 15 min. The lipofectin/DNA mixture was prepared by gently mixing two solutions and incubated for 15 min. For pCI-A58-T-Ag and pRSV-luc-3X κB co-transfected cells, 2 μg T-Ag DNA and 9 μg 3X κB binding elements DNA were mixed in 500 μl Opti MEM for 15 min, respectively, then lipofectin/DNA mixture was prepared by mixing DNA complex and 50 μg lipofectin for another 15 min. HUVECs were incubated with DNA and lipofectin mixture at 37°C in 5% CO_2 for 3 h. At the end of transfection, the culture media were changed to normal EGM allowing cells recovery for 1–2 days followed by the selection media containing G418.

Selection and Cloning

The transfection efficiency was about 5–10% in primary HUVECs. To separate the transfected cells from non-transfected cells, G418 (1 mg/ml; Gibco^{TM} Invitrogen Corporation) was added 2 days after transfection, and the selection medium was renewed once a week. The transfected cells expressing neomycin resistance gene were survival in the selection media while the non-transfected cells were killed by higher concentration of G418. The colonies emerged 2 weeks later, when active growing cells were individually picked using conventional cloning techniques and expanded to mass cultures. The selected HUVECs were cultured in EGM containing G418 500 μg/ml, passages 2–15 were identified by following methods.

Characterization

To investigate endothelial characteristics, phenotype and function were checked.

Expression of T-Ag. Expression of the T-Ag gene product, large T protein in HUVECs lines was assayed by Western blot analysis.

Non-transfected, transfected HUVECs, and COS-7 cells (a large T protein positive cell line using as control) were grown to 80% confluence, harvested in cell lysis buffer on ice. SDS–PAGE was performed and the proteins were electrophoretically transferred to membranes. After being blocked, the membranes were incubated with a polyclonal primary antibody for 1 h at room temperature followed by incubation with anti-mouse Ig antibody-horseradish peroxidase conjugate for large T protein detection. The bound immunocomplexes were visualized byenhanced chemiluminescence (ECL Western Blotting Analysis System, Amersham, Piscataway, NJ).

The intranuclear large T protein was identified by immunofluorescence detection using a Zeiss confocal laser-scanning microscope, as well. In brief, monolayer of non-transfected and transfected HUVECs was seeded on laminin (20 μg/ml, Gibco) coated glass coverslips, respectively. Cells were fixed in 3% paraformaldehyde for 20 min and permiabilized by 0.2% Triton X-100 for 10 min at room temperature. Immunostaining employed primary antibodies against large T protein and secondary FITC-tagged antibody that were used according to the manufacturer's recommendations. DAPI (0.25 μg/ml) staining was performed during the last wash. Images were captured on a Zeiss confocal laser-scanning microscope. FITC was visualized using the 488/510 nM of excitation/emission filter combinations.

Expression of vWF. To confirm the phenotype was preserved after transgene treatment, non-transfected, and transformed HUVECs lines were recognized by anti-vWF primary antibody and FITC linked secondary antibody. Cells were examined with a Zeiss confocal laser-scanning microscope.

Uptake of DiI-Ac-LDL. To evaluate the uptake function of lipoproteins with non-transfected and transfected HUVECs, cultures were incubated with low density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL, Biomedical Technologies, Stoughton, MA) and examined by a Zeiss confocal laser-scanning microscope. Briefly, cells were seeded in p35-mm plastic dishes at density of 1×10^5 per dish, respectively. DiI-Ac-LDL was diluted in normal medium (10 μg/ml) and incubated with cells for 4 h at 37°C. For excitation, the 514-nm line of an argon laser was used, and fluorescence emission 550 nm was collected.

Reporter Gene Assay

Selected and identified HUVECs were cultured in p35-mm plastic dishes at a density of 1×10^5 cells per dish. Monolayers were allowed to attach in EGM. TNFα in a final concentration of 20 nM (R & D system, Inc., Minneapolis, MN) was added for O/N allowing synthesis of luciferase protein. Luciferase activity in cell lysates was measured with a luminometer.

RESULTS

Typical endothelial characteristics can be divided into phenotype and function.

Morphology

Comparing the morphological features observed in the second subculture of transfected and non-transfected HUVECs, both of them exhibited a cobble stone appearance that was similar to non-transfected HUVECs.

Phenotype

Expression of the large T protein

Western Blotting. With the aim of confirming *T-Ag* gene expression in transfected HUVECs lines, HUVECs, transformed HUVECs lines, and COS-7 cells at passage 2 were subjected to immunoblotting with the anti-T-Ag antibody. In HUVECs there is no expression of T-Ag protein (Fig. 1A, lane 2). Both the transformed HUVECs lines and positive control, COS-7 cells clearly showed the presence of large T protein (Fig. 1A, lane 1, 3–5). To further confirm the permanent expression of *T-Ag* gene, Western blotting was repeated at passage 10 (Fig. 1A, lane 6).

Immunocytochemistry Studies. As shown in Figure 1B, large T protein was easily identified by immunofluorescence in green color in nuclei. Meanwhile, a specific nuclear dye, DIPA, visualized nuclei of HUVECs in blue color.

Expression of HUVECs markers, vWF

Immunocytochemistry Studies. Immunofluorescent staining for presence of vWF was performed. Both non-transfected and transfected HUVECs expressed Factor VIII related protein located in cytosol (Fig. 2).

Function

Uptake function of HUVECs for lipids

Immunocytochemistry Studies. HUVECs were brightly stained in red color indicating the Ac-LDL could be uptaken into cells. The

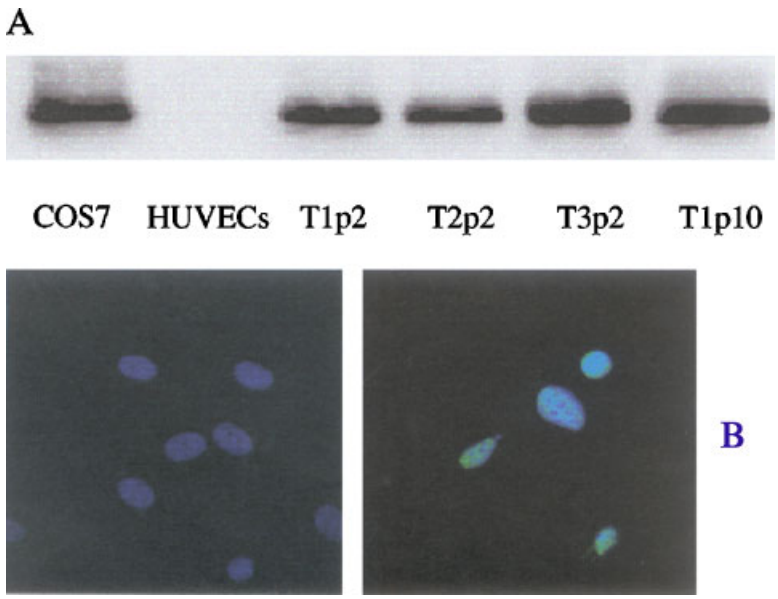

Fig. 1. Expression of *T-Ag* gene. **A**: Western blotting for large T protein in cell lysates of non-transfected and transfected HUVECs. **Lane 1**: COS-7 cell lysate containing large T protein loaded as positive control. **Lane 2**: Non-transfected HUVECs. **Lanes 3–5**: Expression of large T protein in three HUVECs monoclonal lines that were co-transfected with T-Ag and NF-κB binding elements gene at passage 2 (T1p2, T2p2, and T3p2: transfected HUVECs line 1–3 at passage 2). **Lane 6**: Recheck *T-Ag* gene expression at passage 10 of the first co-transfected cell line (T1p10: transfected HUVECs line 1 at passage 10). **B**: Confocal images. Intranuclear antigen encoded by T-Ag of SV-40 in transfected HUVECs. ×400. Non-transfected HUVECs without *T-Ag* gene expression (**left**). DIPA staining showed nuclei of HUVECs. HUVECs transfected with *T-Ag* gene. DIPA staining showed nuclei of HUVECs in blue color. Green color showed T-Ag located in the nuclei (**right**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

fluorescence was predominantly punctated with a perinuclear distribution. In *T-Ag* gene transfected HUVECs, and in T-Ag and NF-κB binding elements gene co-transfected HUVECs, similar style images were observed (Fig. 3).

Reporter Gene Assay

Measurement of NF-κB activation. To determine the stage in NF-κB activation affected by TNF-α, three of monoclone co-transfected

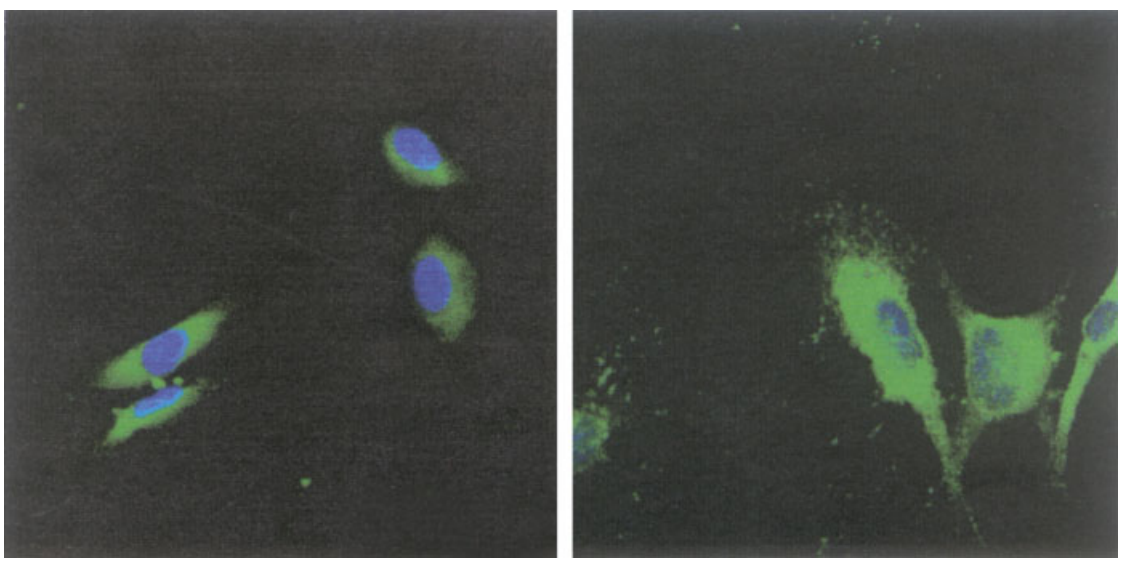

Fig. 2. Expression of vWF. Confocal images. Cytosolic Factor VIII related protein (vWF) was identified by anti-vWF primary antibody and FITC linked secondary antibody in non-transfected HUVECs (**left**) and transfected (**right**). Non-transfected HUVECs: DIPA staining indicated nuclei of HUVECs in blue color. Localization of vWF in cytosol in green color. Transfected HUVECs: green color showed T-Ag located in the nuclei and vWF in cytosol. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

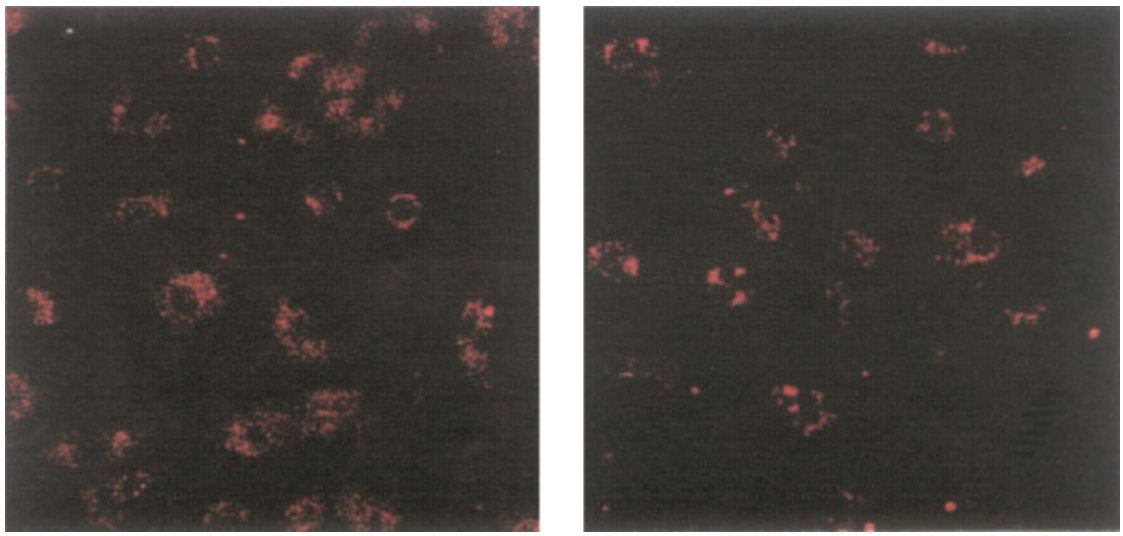

Fig. 3. Up took of DiI-labeled ac-LDL in transfected and non-transfected HUVECs. Cytosolic fluorescence signal showed DiI labeled Ac-LDL in red color. Similar style of non-transfected HUVECs (**left**) and transfected (**right**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cell lines were treated with TNF-α. Figure 4 showed that TNF-α significantly increased NF-κB transactivation comparing with untreated cells.

DISCUSSION

A cell line should be stable expression of at least one or two specific endothelial markers characterizing the cells as EC, such as presence of WP-bodies, vWF-expression, Ac-LDL uptake, ACE activity, cobblestone morphology. It also should be stable expression of the experimentally relevant markers [Bouis et al., 2001].

In the present study, we successfully transfected HUVECs using plasmids containing T-Ag and NF-κB binding elements genes. The cells retained their EC phenotypic characteristics. Morphologically, a monolayer of these cell lines displayed a cobblestone-like appearance at confluence. Their morphology was similar to that described by others for cultured endothelium [Lewis et al., 1973].

Expression of simian virus 40 early genes has been shown to transform cells and allow establishment of permanent cell lines [Asselin and Bastin, 1985]. The maintenance of the immortalized phenotype is dependent on the presence

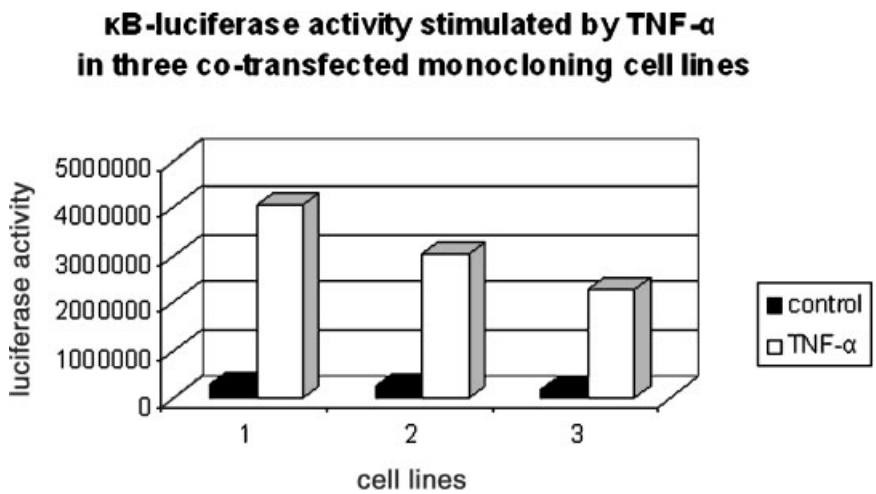

Fig. 4. Reporter gene assay. Comparing with non-transfected HUVECs (violet), three monocloned transfected cell lines showed significant higher luciferase activity after stimulated by TNF-α (red). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of the large T protein, involving gene activation, protein–protein interaction with anti-oncogene products.

EC cultures secrete a protein that was apparently specific for EC, vWF also known as Factor VIII-related antigen. Weibel–Palade-bodies (WP-bodies) stored large amounts of vWF that could quickly be released upon activation of the cells. vWF was a large adhesive glycoprotein synthesized in EC. In the blood it served as a stabilizing carrier for Factor VIII with which it circulated as a complex. As another means of identifying our transfected HUVECs lines as authentic EC was that this marker was observed by a Zeiss confocal laser-scanning microscope. Similar with non-transfected HUVECs, T-Ag only or T-Ag and NF-κB binding elements genes co-transfected HUVECs lines showed bright fluorescent in a perinuclear pattern.

Functionally, the ability of taking proteins from culture medium of HUVECs lines was also checked by adding DiI-Ac-LDL to medium. DiI was a highly lipophilic molecule that could be noncovalently incorporated into lipoproteins and thus has no effect on surface change. Once cells took up a lipoprotein that containing DiI, the later was acted upon by lysosomal enzymes and the DiI accumulates in lysosomal membranes [John et al., 1984]. So that DiI-Ac-LDL was potentially useful in situations where EC identifications was desired. As we had known that the receptor-mediated uptake of LDL by cells has been studied in detail [Goldstein and Brown, 1977]. An alternative pathway for the metabolism of chemically modified lipoproteins has also been described and has been termed the "scavenger cell pathway" of LDL metabolism. In this study, we have demonstrated that the Ac-LDL was taken up by non-transfected HUVECs and transfected HUVECs, as well. Furthermore, the transformed HUVECs were brilliantly fluorescent similar to non-transfected HUVECs indicating the ability of uptaking Ac-LDL of transformed cells was retained after transfection. The basic function of endothelium was preserved after transfection. The cell lines could be used for research as non-transfected cell.

The pRSV-luc-3XκB vector contained the κ enhancer element within the promoter region. Once activated, endogenous NF-κB binds to the κ enhancer element and activated luciferase expression. In this article TNF-α, a typical cytokine increasing the activity of NF-κB was used to treat the three transfected cell lines O/N, respectively. The higher luciferase reporter gene activity was seen in the cell lysates on the next day. Similar to TNF-α, drugs affecting on NF-κB activation would make a change in luciferase activity as well. Therefore, people may use these cell lines to learn if a drug acts on transcriptional level by simply performing a luciferase assay.

CONCLUSION

In summary, these properties make *T-Ag* gene transfected or T-Ag and NF-κB binding elements gene co-transfected HUVECs a good in vitro model for study. They might be used to check reporter gene activity directly. It might be a useful tool to screen drugs acting on transcription level.

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