Biological effects of eukaryotic recombinant plasmid pReceiver-M61-BAI-1 transfection on T24 cells and HUVECs

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Abstract. The aim of the current study was to investigate the biological effect on T24 cells and human umbilical vein endothelial cells (HUVECs) of transfection with brain-specific angiogenesis inhibitor-1 (BAI-1). The recombinant plasmid pReceiver-M61-BAI-1 was transfected into human superficial bladder tumor cells (T24) and HUVECs, in parallel with the vector control. mRNA and protein expression levels of BAI-1 were then detected by quantitative polymerase chain reaction (qPCR) and western blotting, respectively. Cell apoptosis of T24 cells and HUVECs prior and subsequent to transfection with BAI-1 was analyzed by flow cytometric analysis. Proliferation of T24 cells and HUVECs prior and subsequent to transfection of BAI-1 was assessed by the MTT method. T24 cells and HUVECs transfected with pReceiver-M61-BA1-1 were classed as the experimental group; T24 cells and HUVECs transfected with p-Receiver-M61 were the control group. qPCR and western blotting methods confirmed that there was positive expression of BAI-1 in T24 cells and HUVECs transfected with pReceiver-M61-BAI-1, however BAI-1 was not expressed in T24 cells and HUVECs transfected with pReceiver-M61. The results of the MTT assay demonstrated that absorbance was markedly reduced in HUVECs at 12, 48 and 72 h subsequent to transfection with pReceiver-M61-BAI-1 when compared with that of the control group and in T24 cells transfected with p-Receiver-M61-BAI-1. Furthermore, flow cytometry results also indicated that the apoptotic rate of HUVECs transfected with p-Receiver-M61-BAI-1 was significantly increased compared with that of the control group and T24 cells transfected with p-Receiver-M61-BAI-1. BAI-1 was observed to markedly inhibit the proliferation of vascular endothelial cells *in vitro*, however, no direct inhibition by BAI-1 was observed in T24 cells. In conclusion, BAI-1 is suggested to be a potential novel therapautic target for the inhibition of tumor neovascularization.

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Introduction

At present, superficial bladder cancer is stratified by differentiation grade and stage into three groups of different risk profiles (Ta G1-2 vs. T1 G1-2 vs. Tis/T1 G3). At present, the standard therapy is fractionated transurethral resection (1). Although the rate of mortality with this therapy is low, the recurrence rates are 50-70%, of which 10-20% cases develop into muscle-infiltration bladder cancer, and the five-year survival rate was less than 50% (2-6). It is necessary to have post-operative therapy and closely follow up patients with bladder cancer following surgery. Intravesical therapy is an effective measure used to reduce the recurrence rate and the aggression of bladder cancer. When administered to high risk patients with non-infiltration bladder tumors, it was previously reported that intravesical Bacillus Calmette-Guerin vaccine exhibited in a clear treatment effect and a reduced recurrence rate of bladder cancer, however had no effect of reducing tumor aggression (7,8). In addition, bladder tumors that are resistant to this vaccine are more likely to exhibit recurrence and develop into infiltrated tumors (9). Thus, it was considered important to investigate novel treatment strategies in intravesical therapy in the current study.

When a novel p53-inducible gene was identified, a target gene that was specifically expressed in the brain and that inhibited *in vivo* neovascularization induced by basic fibroblast growth factor (bFGF) in the rat cornea was additionally identfied, which was named brain-specific angiogenesis inhibitor-1 (BAI-1) (10). However, it has now been observed that BAI-1 is present not only in brain tissue, however additionally in the colon, stomach, lung and pancreas. Notably, Fukushima *et al* (11) demonstrated that the levels of BAI-1 were markedly lower in colon cancer tissue samples when compared with normal colon tissues, and that there was a correlation between BAI-1 levels and malignancy of the tumor. Izutsu *et al* (12) additionally identified that BAI-1 was present in renal cell carcinoma samples, and that the BAI-1 levels were increased in normal renal tissue compared with

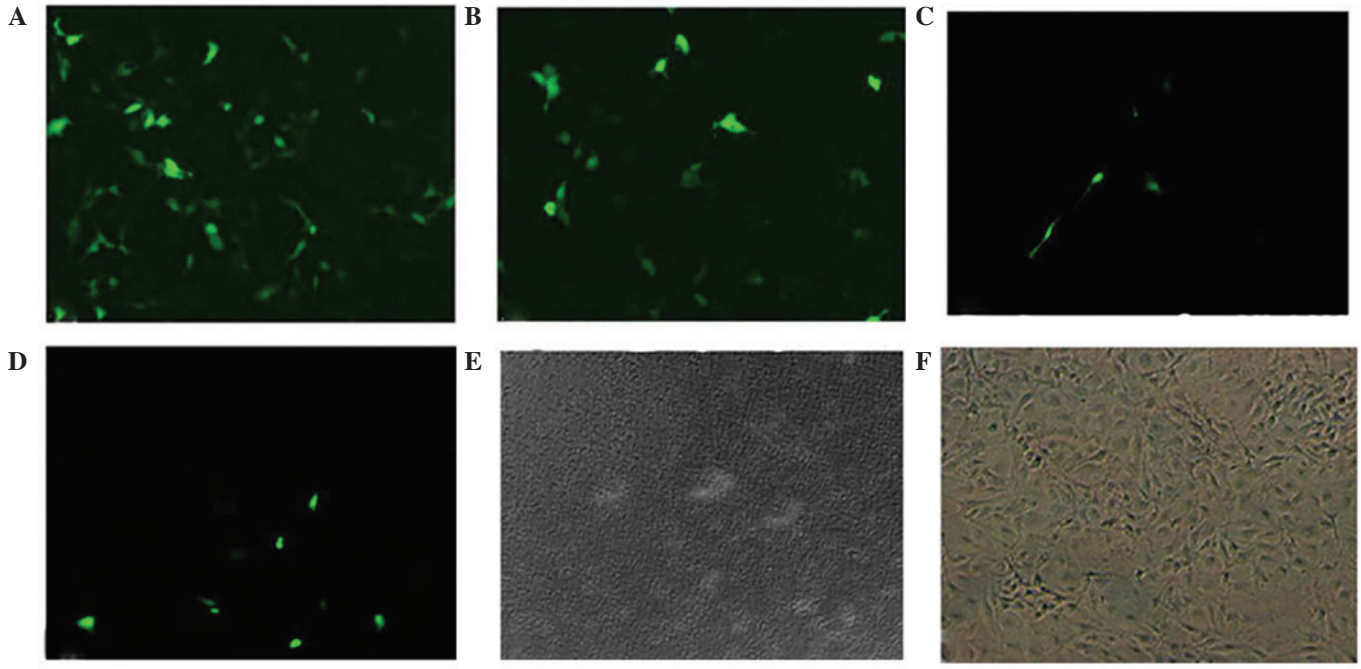

Figure 1. (A) T24 cells and (B) HUVECs subsequent to transfection with pReceiver-M61 (magnification, x100). (C) T24 cells and (D) HUVECs subsequent to transfection with pReceiver-M61-BAI-1 (magnification, x100). (E) T24 cells under a magnification of x40 using a light microscope. (F) HUVECs under a magnification of x100 using a light microscope. HUVECs, human umbilical vein endothelial cells; BAI-I, brain-specific angiogenesis inhibitor-1.

renal cell cancer tissue. BAI-1 encodes a seven-span transmembrane protein, containing five thrombospondin type-1 (TSP-1) repeats that inhibited *in vivo* neovascularization induced by bFGF through interactions between its receptors and CD36 (13). In the current study, the effects of BAI-1 plasmid transfection on T24 cells and human umbilical vein endothelial cells (HUVECs) were investigated, with the aim to provide experimental evidence that would aid in the development of novel therapeutic targets for the treatment of bladder cancer.

Materials and methods

Reagents and chemicals. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from EMD Millipore (Billerica, MA, USA). Spectrophotometer, flow cytometer, and micro-spectrophotometer were purchased from Beckman Coulter, Inc. (Brea, CA, USA). The fluorescence microscope was purchased from Olympus (CX31; Olympus Corporation, Tokyo, Japan). The polyclonal rabbit anti-BAI-1 (1:200; ab135907), polyclonal rabbit anti-β-actin (1:200; ab8227), goat anti-rabbit secondary antibody (1:1,000; ab97080) were obtained from Abcam (Cambridge, UK). All other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

Establishment of the p-Receiver-M61-BAI-1 plasmid. According to the design principles of establishing an open reading frame plasmid, the NCBI website was searched for BAI-1 mRNA (NM-001701). The mRNA length of BAI-1 was 5,535 bp, and a BAI-1 plasmid labelled with green fluorescent protein was established on the basis of BAI-1 primer sequences outlined by Kudo *et al* (14).

0BAI-1-siR-Top, GGACTTTAGAAGCCGTTGCTGCCC TCTCTGTCACCTGAAGCGGGGCCCTCTCCCATCCCA; BAI-1-siR-Bot, ATTTTTTCTCTCCTTTTCTTTTCTTCA ATAAAAAGAATTAAAAACCCAAAAAAAA. BAI-1, forward 5'-GCGGTAGGCGTGTACGGT-3' and reverse 5'-AGCAGTCCCCAAGTCAGT-3'. The concentration of the plasmid was detected using a micro-spectrophotometer, pReceiver-M61-BAI-1 plasmid concentration was 180 ng/μl. The expression of pReceiver-M61-BAI-1 was analyzed using agarose gel electrophoresis. The electrophoretogram indicated that sequences of recombinant plasmid pReceiver-M61-BAI-1 were as expected and the plasmid had been established correctly.

Transfection of p-Receiver-M61-BAI-1 into T24 cells and HUVECs. T24 human superficial bladder tumor cells and HUVECs were provided by the Tianjin Institute of Department of Urinary Surgery (Tianjin, China). The HUVECs and T24 cells were grown until they reached the logarithmic phase. All cells were seeded into 6-well plates and cultured in a humidified incubator at 37˚C under conditions of 5% CO_2. Cells were prepared for transfection subsequent to reached 50-60% confluence. T24 cells and HUVECs were divided into the pReceiver-M61-BAI-1 group and p-Receiver-M61 control vector group, and each group had three identical wells. RPMI 1640 medium containing 10% fetal calf serum (1 ml) was added into all wells 8 h subsequent to transfection, then the cells were cultured in a humidified incubator at 37˚C under 5% CO_2 for 15 h. Each well was photographed, subsequent to which the cells were collected after 48 h. Protein and gene expression levels of BAI-1 were detected by western blot analysis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

RT-qPCR. Total RNA was reverse transcribed to cDNA by a Reverse Transcription kit (Roche Diagnostics, Basel Switzerland). RT-qPCR was performed using an Applied Biosystems 7900HT thermal cycler, with a 20 μl PCR reaction mixture containing 10 μl of 2X LightCycler 480 SYBR Green I Master mix (Roche Diagnostics). The following primers were used: BAI-1, forward 5'-CCGCTGTGTTTCCATTGACTA-3' and reverse 5'-ACCACAAACACGGATGCTTCA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-GAAGGTCGGAGTCAACGGAT-3' and reverse 5'-CTGGAAGATGGTGATGGGATT-3'. The qPCR cycling conditions were as follows: 95˚C for 10 min followed by 40 cycles of 95˚C for 10 sec, 58˚C for 20 sec and 72˚C for 20 sec, followed by 72˚C for 5 min. qPCR was used to measure the gene expression levels of BAI-1. The results were normalized using the $2^{-\Delta\Delta Cq}$ method (15).

MTT assay to detect the effects of BAI-1. Group A, Normal T24 cells and HUVECs cultured for 12, 48 and 72 h; group B, T24 cells and HUVECs with pReceiver-M61 cultured for 12, 48 and 72 h; group C, T24 cells and HUVECs with pReceiver-M61-BAI-1 cultured for 12, 48 and 72 h. HUVECs and T24 cells in the logarithmic growth phase were collected and made into a cell suspension with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA), then were plated into 96-well plates ($5x10^3$/well) in a humidified incubator overnight at 37˚C under 5% CO_2. Subsequent to 12, 48 and 72 h incubation, 10 μl MTT solution/well was added, and culture was continued for 4 h. Subsequently, 100 μl dimethyl sulfoxide was added, then the contents of the wells were dissolved using a vibrating machine for 10 min. The optical density (OD) value of each well was detected by an enzyme-linked determining instrument. Each experiment was repeated three times. Inhibition rate = (control group OD - experiment group OD)/(control group OD - blank group OD) x 100%.

Flow cytometry assay to detect T24 cell and HUVEC apoptosis subsequent to transfection. Cells in the logarithmic growth phase were plated into 6-well plates overnight and transfected with the BAI-1 plasmid. Groups were as the same as for those used in the MTT assay. Susbequent to culture for 12, 48 and 72 h, $1x10^6$ cells were collected and centrifuged at 300 x g for 5 min at room temperature, followed by being washed twice with phosphate-buffered saline. Subsequent to fixing in cold 70% ethanol, the plates were detected by flow cytometry.

Western blot analysis. T24 cells and HUVECs were transfected with pReceiver-M61-BAI-1. The cell lysates were cleared by centrifugation at 12,000 x g for 30 min at 4˚C. The harvested cells were suspended in phohsphate-buffered saline containing protease and phosphatase inhibitors and homogenized. The homogenates were centrifuged at 14,000 rpm for 40 min at 4˚C, and the resultant supernatant fractions were used for immunoblotting. A bicinchoninic acid assay was used to quantify the protein. Samples containing 50 μg of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Following blocking with 5% (w/v) non-fat dry milk in Tris-buffered saline and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies: BAI-1 and β-actin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), at 1:200 at 4˚C overnight. Following three washes with TBST, membranes were incubated the secondary antibody for 1 h at room temperature. Western blots were quantified with HP-Scanjet 550c and analyzed by UN-SCAN-IT software (Silk Scientific, Orem, UT, USA).

Statistical analysis. Statistical analysis was performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA) and all results were presented as the mean ± standard deviation. Student's t-test was used to compare data between the

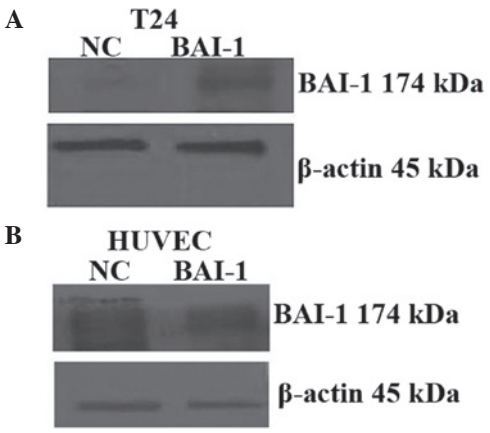

Figure 2. Protein expression of BAI-1 levels in (A) T24 cells and (B) HUVECs by western blot analysis. HUVECs, human umbilical vein endothelial cells; NC, negative control; BAI-I, brain-specific angiogenesis inhibitor-1.

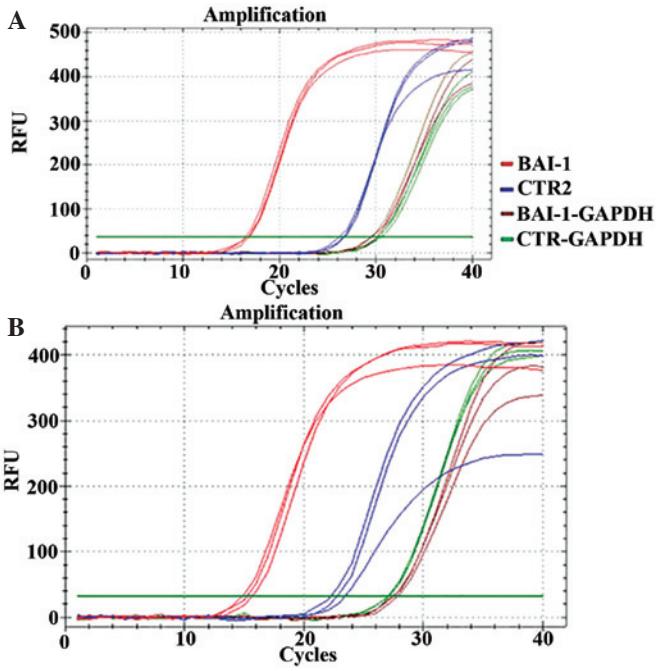

Figure 3. Quantitative polymerase chain reaction analysis of (A) HUVEC-BAI-1 and (B) T24-BAI-1 cells. HUVEC, human umbilical vein endothelial cell; BAI-1, brain-specific angiogenesis inhibitor-1; CTR, control plasmid; GAPDH, internal control, glyceraldehyde 3-phosphate dehydrogenase; RFU, relative fluorescence units.

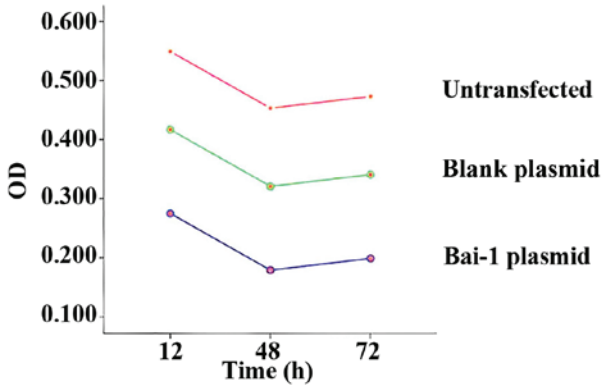

Figure 4. OD values at different time-points in HUVECs subsequent to transfection with different plasmids. OD, optical density; HUVECs, human umbilical vein endothelial cells; BAI-I, brain-specific angiogenesis inhibitor-1.

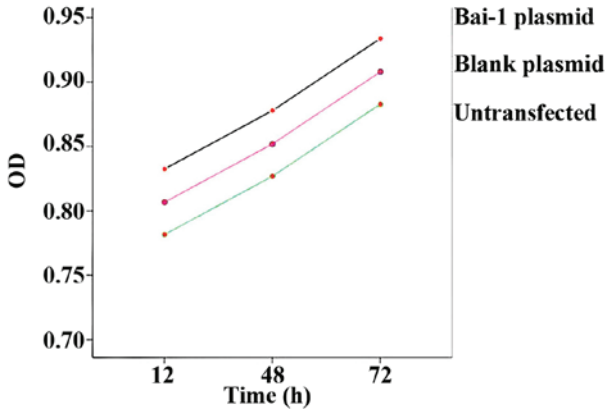

Figure 5. OD values at different time-points in T24 cells subsequent to transfection with the plasmids. OD, optical density; BAI-I, brain-specific angiogenesis inhibitor-1.

groups and the χ^2 test was used for two-sample comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Western blotting analysis to detect the protein expression levels of BAI-1 in T24 cells and HUVECs. pReceiver-M61-BAI-1 and pReceiver-M61 labelled with green fluorescent protein were transfected into T24 cells and HUVECs (Fig. 1). Plasmids were identified to have been successfully transfected through the detection of the protein expression levels of BAI-1 in T24 cells and HUVECs subsequent to transfection with pReceiver-M61-BAI-1. T24 cells and HUVECs transfected with p-Receiver-M61-BAI-1 were identified to express BAI-1 protein, however no expression of BAI-1 was observed in T24 cells and HUVECs transfected with pReceiver-M61 (Fig. 2).

qPCR assay to detect the gene expression of BAI-1 subsequent to transfection in T24 cells and HUVECs. Plasmids were identified to have been successfully transfected through the detection of the gene expression of BAI-1 in T24 cells and HUVECs subsequent to transfection with pReceiver-M61-BAI-1 (Fig. 3).

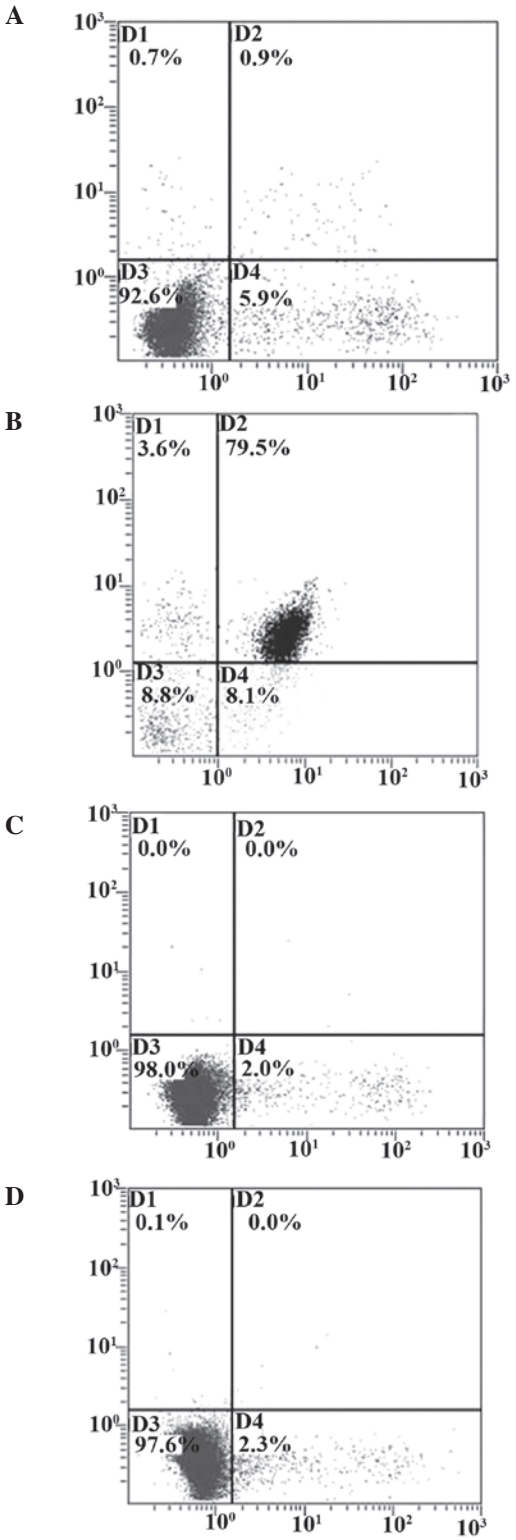

Figure 6. The apoptotic rate of HUVECs and T24 cells, following 72 h culture. (A) pReceiver-M61-BAI-1 transfection of T24 cells. (B) pReceiver-M61-BAI-1 transfection of HUVECs cells. (C) pReceiver-M61 transfection of T24 cells. (D) pReceiver-M61 transfection of HUVECs cells. BAI-I, brain-specific angiogenesis inhibitor-1; HUVECs, human umbilical vein endothelial cells.

Effect of BAI-1 on the proliferation of T24 cells and HUVECs. Subsequent to transfection of BAI-1, it was identified that BAI-1 inhibited the proliferation of HUVECs. In addition, it was demonstrated that the longer the transfection duration,

Table I. OD values at different time-points subsequent to plasmid transfection into human umbilical vein endothelial cells.

		Group					
Time	n	BAI-1	Negative plasmid	Normal	Sum	F-value	P-value
12 h	48	0.39±0.068	0.35±0.062	0.49±0.070	0.41±0.086		
48 h	48	0.16±0.016	0.33±0.057	0.46±0.071	0.32±0.136		
72 h	48	0.09±0.022	0.40±0.068	0.52±0.060	0.34±0.190		
						12.523	0.000
Sum	144	0.22±0.138	0.36±0.068	0.49±0.069	0.36±0.149	91.934	0.000

The OD values were significantly different at the different time-points (F=12.523, P=0.000), with the OD value observed to be significantly greater at 12 h when compared with that of 48 and 72 h. Statistical significance was also observed between the different plasmids (F=91.934, P=0.000); OD values were observed to be the highest in the normal group, and the lowest in the BAI-1 group. OD, optical density, BAI-1, brain-specific angiogenesis inhibitor-1.

Table II. OD values at different time-points subsequent to plasmid transfection into T24 cells.

		Group					
Time	n	BAI-1	Negative plasmid	Normal	Sum	F-value	P-value
12 h	48	0.83±0.163	0.80±0.111	0.79±0.121	0.81±0.132		
48 h	48	0.84±0.155	0.84±0.102	0.88±0.119	0.85±0.126		
72 h	48	0.90±0.142	0.85±0.163	0.98±0.227	0.91±0.185		
						5.185	0.007
Sum	144	0.86±0.153	0.83±0.127	0.88±0.178	0.86±0.154	1.308	0.274

Normal T24 cells vs. T24 cells with pReceiver-M61 (P<0.01). Significant differences were identified between the OD values at different time-points (F=5.185, P=0.007), with significance observed between the 12 and 72 h time-points. No significant differences were identified among the different plasmids (F=1.308, P=0.274). OD, optical density; BAI-1, brain-specific angiogenesis inhibitor-1.

the greater the inhibition rate was (P<0.01; Table I; Fig. 4), however, there was no significant difference prior and subsequent to transfection in T24 cells (P=0.274; Table II; Fig. 5). Furthermore, there was no significant difference observed between normal HUVECs and HUVECs transfected with pReceiver-M61. These results suggest that BAI-1 significantly inhibited growth of HUVECs, however with no clear effect on T24 cells.

Detection of cell apoptosis subsequent to transfection with BAI-1 in T24 cells and HUVECs. The pReceiver-M61 and pReceiver-M61-BAI-1 plasmids were transfected into T24 cells and HUVECs, then were detected by flow cytometry. Flow cytometry results indicated that BAI-1 resulted in an increase in HUVEC apoptosis 72 h subsequent to transfection, however no clear effect was observed in T24 cells (Fig. 6).

The apoptotic rate of HUVECs transfected with pReceiver-M61-BAI-1 was 79.5% 72 h subsequent to transfection, however the apoptotic rate of T24 cells transfected with pReceiver-M61-BAI-1 was 0.9%. No significant difference in the apoptotic rates of HUVECs transfected with pReceiver-M61 and T24 cells transfected with pReceiver-M61 was observed.

Discussion

BAI-1 is located in 8q24.3, is 80.99 kb and contains 30 exons and a minimum of one functional p53-binding site within an intron. BAI-1 encodes a 1,584-amino-acid product (10) and is a member of the adhesion-G protein-coupled receptor (GPCR) family of receptors (16). *In vitro*, TSP-1 has been identified to inhibit the migration of endothelial cells and angiogenesis mediated by CD36 (17,18). Dawson *et al* (19) identified that IgG antibodies against CD36 and glutathione-S-transferase-CD36 fusion proteins that contain the TSP-1 binding site blocked the ability of intact TSP-1 and its active peptides to inhibit the migration of cultured microvascular endothelial cells. In addition, transfection of CD36-deficient HUVECs with a CD36 expression plasmid resulted in them becoming sensitive to TSP-1 inhibition of migration and tube formation. Thus, TSP-1 repeats of BAI-1 had obviously effect of inhibition on proliferation of vascular endothelial cells. Hatanaka *et al* (20) examined gene expression of BAI-1 in 48 lung adenocarcinoma specimens by qPCR and vascular density was detected by immunohistochemistry using the anti-CD34 monoclonal antibody. They confirmed that BAI-1 gene expression was detected in 38 out of the 48 pulmonary adenocarcinoma samples (79.2%), and the vascular

number and measurement area were significantly reduced in the BAI-1-positive pulmonary adenocarcinoma samples (19.3+/-4.4/μm^2 and 1.7+/-0.6%) as compared with those in the BAI-1-negative carcinomas (75.5+/-42.7/μm^2 and 5.5+/-1.5%). These results indicated that BAI-1 expression may inhibit stromal vascularization in lung adenocarcinomas, however how angiogenesis is inhibited remains unclear. Furthermore, Yoon *et al* (21) demonstrated that the extracellular region of BAI-1 (BAI-1-ECR) could inhibit angiogenesis. Rabbits were injected with the BAI-1-ECR gene or empty vector two or three times at 1 week intervals beginning 1 week subsequent to debridement and the results indicated that BAI-1-ECR gene delivery effectively reduced experimental corneal neovascularization. In addition, Kaur *et al* (22) demonstrated that BAI-1 was proteolytically cleaved at a conserved GPCR proteolytic cleavage site, releasing its 120 kDa extracellular domain. This secreted fragment was termed vasculostatin as it inhibited migration of endothelial cells *in vitro* and markedly reduced *in vivo* angiogenesis. The site of hydrolysis was the site of proteolytic cleavage in conservative GPCRs (23). However, it remains unclear which enzymes are able to recognize this site; with a previous study indicating that BAI-1 provides a site to perform proteolytic processing and release proteins that inhibit angiogenesis. Previous studies identified that there was an association between tumor growth and the concentration of vascular inhibiting fragments; in addition, vascular inhibiting fragments were observed to be associated with tumor prevention.

Therefore, it is suggested that BAI-1 may be considered as a tumor suppressor gene and has been demonstrated to exhibit low expression in cancerous tissues. In the current study, the BAI-1 over-expression plasmid was transfected into T24 cells and HUVECs in order to observe the alterations to T24 cells and HUVECs. pReceiver-M61 was a eukaryotic expression vector labelled with green fluorescence. Transfection efficiency was calculated through detection of fluorescent intensity. The results indicated that green fluorescent protein expression was disperse in T24 cells and HUVECs, observed under the microscope. This indicated that BAI-1 was located in the cytoplasm. In addition, protein and gene expression of BAI-1 was confirmed in T24 cells and HUVECs subsequent to transfection, observed through qPCR and western blotting. In addition, it was identified that compared with HUVECs transfected with p-Receiver-M61, proliferation of HUVECs transfected with p-Receiver-M61-BAI-1 was significantly inhibited, observed using the MTT method ($P<0.05$). The current study observed 72 h subsequent to transfection, and it was identified that with time after transfection, the concentration of p-Receiver-M61-BAI-1 was reduced. In addition, it was identified that BAI-1 had no direct inhibitory effect on the proliferation of T24 cells *in vitro*. There was no significant difference between T24 cells transfected with p-Receiver-M61-BAI-1 and those transfected with p-Receiver-M61 ($P>0.05$). Furthermore, flow cytometry was used to detect the apoptosis of T24 cells and HUVECs over a 72 h time period subsequent to transfection of the p-Receiver-M61-BAI-1 and p-Receiver-M61 plasmids. The results indicated that BAI-1 increased the apoptosis of HUVECs, however did not affect that of T24 cells. Thus, it is suggested that BAI-1 inhibited proliferation of vascular endothelial cells to inhibit tumor growth, however had no direct effect on T24 cell death.

In conclusion, BAI-1 is a member of the adhesion-GPCR family of receptors. Numerous diseases are associated with GPCRs, which are the targets of approximately 40% of drugs. Thus, BAI-1 is suggested to be a potential novel therapautic target for the inhibition of tumor neovascularization.

Acknowledgements

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