RRPC90091210 (7 .P)

行政院國家科學委員科會專題研究計畫

探討在Thy-1血管增生過程中所扮演的角色(3/3)

計畫編號：NSC 90-2320-B-038-032
執行期限：九十年八月一日至九十一年七月三十一日
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一、Introduction

Duirng the first two years of this project, we have established the primary culture systems of endothelial cells including rat aortic endothelial cells, human umbilical venous cells (HUVEC) and human dermic microvascular endothelial cells (HDMVEC). Thymidine incorporation was used to show the DNA patterns of the cell cycle of endothelial cells. We have also successed in the immunstaining for Thy-1 protein. Moreover, administration of the anti-Thy-1 antibody induced a dose-dependent increase of thymidine incorporation in rat aortic endothelial cells and HDMVEC. Treatment of the rat aortic endothelial cells with Thy-1 antisense oligonucleotide induced a dose-dependent inhibition of thymidine incorporation of rat aortic endothelial cells. Thy-1 overexpressed rat aortic endothelial cell grew faster than the vector-transfected rat aortic endothelial cell. The aim of the third year study was focused on the transfection of HUVEC and NIH3T3 cells with human Thy-1 cDNA and evaluation of Thy-1 effect on the growth of HUVEC and NIH3T3 cells. The results shows as follows.

of vascular endothelial cells.

Materials and Method

Human Thy-1 cDNA was obtained from EST clone and cloned into Lamfmid vector via the cloning sites of Hind III and Not I. For overexpression of human Thy-1 in mammalian cell lines, the full length of Thy-1 was subcloned to the expression vector, pcDNA3.1(+) (Invitrogen) through the same restriction sites as given above and the expression is driven by CMV promoter. The problems associated with human Thy-1 antibodies (i.e. lack of specificity and appearance of multiple bands) were encountered in commercially available Human Thy-1 antibodies. Therefore, Thy-1 gene was alternatively subcloned to fusion protein vector pcDNA3-HA (Invitrogen). The vector pEGFP-N3 purchased from Clontech was used to co-transfect with the construct of pcDNA3-HA-Thy1 for verification of efficiency of transfection in cell culture, which could be observed in an inverted fluorescent microscope. The adenoviral vectors pShuttle-CMV and pAdEasy-1 and E.coli strains BJ5183 and XL-10 Gold cells were kindly provided by Dr. Lin Yi-Ling (Academia Sinica)

Calcium phosphate transfection: HUVEC or NIH3T3 were cultured at 10 cm2 petri-dish to a 80 to 90% confluence and flesh medium was added to cell culture 1-2 hr prior to transfection. Fifty μl of 2.5 M CaCl2 were added to DNA mixture containing 30 μg of pcDNA3-HA-Thy-1 (empty vector used in the control group) and 5 μg of pEGFP-N3 in an aqueous solution, and then mixed with 500 μl of 2-fold HeBS buffer followed by incubation for 15-30 min at room temperature. The mixture was gently added to cell culture with slight rotating the petri-dish for mixing and then cells were incubated for 12-16 hr at 37 oC. Following the incubation, cells were washed 2 times with PBS and added with flesh medium to incubate another 36-40 hr at 37 oC.

Western blot analysis: To prepare whole cell lysates, cells were washed twice with ice-cold PBS, detached by cell scraper in PBS, and then subjected to centrifugation. The resulting pellet was resuspended in RIPA buffer with 1 mM PMSF and kept in ice for 30 min with constant votex every 5 min. The soluble fraction was separated from insoluble part via centrifugation for 30 min at 12,000 RPM. Fifty μg of protein from cell lysates were resolved by SDS gel electrophoresis, transferred to Hybond-P membrane and probed using a mouse anti-Thy-1 monoclonal antibody or mouse anti-HA monoclonal antibody at 1:1,000 dilution or a concentration of 1 μg/ml, respectively and a secondary anti-mouse IgG antibody conjugated with alkaline phosphatese. Signal was dected using BCIP/NBT phosphatase substrate.

MTT assay

Number of viable cells was determined based on the activity of mitochondrial dehydrogenase to reduce MTT to fromazan. Following cotransfection of pcDNA3-HA-Thy1 and pEGFP for 24 hr, cells were plated in 24-well plates at a density of 1x104 cells/well and monitored for 4 days. MTT was then added into each well and incubation continued for 4 hu in culture. The formazan formed was dissolved by overnight incubation with 10 mM HCl containing 10% SDS, and then measured at wavelength of 530 nm.

Construction of adenovirus carrying human Thy-1 gene

The HindIII/NotI restriction fragment of Thy-1 was obtained from the construct of pcDNA3.1 (+)-Thy1 and the 3í end of NotI site in the Thy-1 gene was filled in by Klenow fragment to ligate into the HindIII/EcoRV sites of the pShuttle-CMV vector. The resultant plsmid was linearized with PmeI and cotransformed with the adenoviral backbone vector pAdEasy-1 in Escherichia coli BJ5183 cells by electroporation. Homologous recombinants containing Thy-1 cDNA were detected by restriction endonuclease digestion and agarose gel electrophoresis. Recombinant Thy-1 adenovirus (AdThy-1) was then transformed into E.Coli XL-10-Gold cells for large-scale amplification. The PacI-digested then transformed into E.coli XL10-pAdThy-1 was then transfected in mammalian 293 cells. The empty adenovirus was used as a control and was expanded and purified.

Results

Transfection of cDNA into human primary cell cultures is always a big challenge for the laboratory. In the present study, we have succeeded in the human Thy-1 cDNA construct (see attached Figure) and transfection of this cDNA into HUVEC and NIH3T3 cells. The expression of human Thy-1 protein in the transfected HUVEC and NIH3T3 was demonstrated by Western blot analysis (see attached Figure). The transfection of human Thy-1 cDNA into NIH3T3 cells increased cell growth rate (Figure 3). Although our study of the Thy-1 transfection effect on the HUVEC is still on going, the finding from the human Thy-1 transfected NIH3T3 cells was consistant with our previous findings showing that using the anti-Thy-1 antibody which activates Thy-1 dose-dependently increased thymidine incorporation, and using the anti-thy-1 antisense oligonucleotide to neutralize the Thy-1 expression dose-dependently decreased thymidine incorporation. Taken together, the results from these series studies suggest that Thy-1 protein, which is expressed in the angiogenic microvascular endothelial cells but not normal resting endothelial cells, might participate in the activating of the process of angiogenesis.

Transfect NIH3T3 with HA-Thy-1

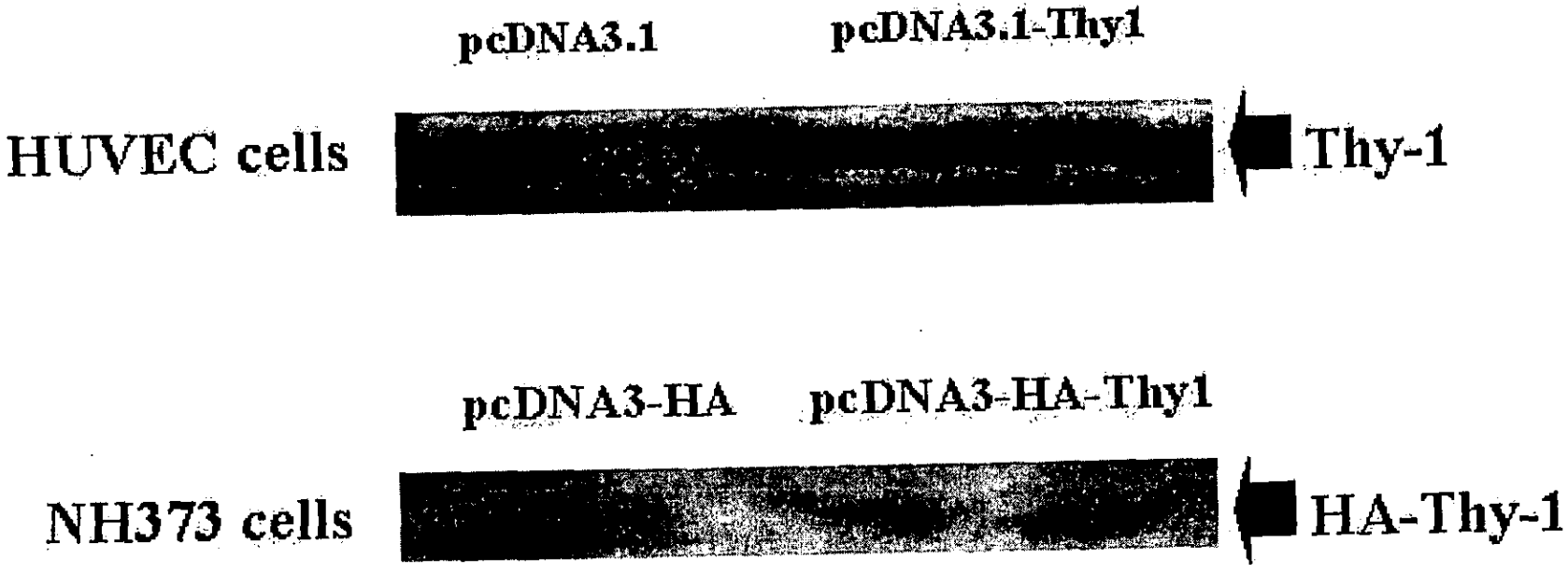
Control	2.5M $CaCl_2$	50 μl
	1ug/ul pSP72	30 μl (30 μg)
	1.54ug/ul pEGFP	3.2 μl (5 μg)
	water	417 μl
	2X HeBS	500 μl
Experiment	2.5M $CaCl_2$	50 μl
	1ug/ul pSP72	30 μl (30 μg)
	5.56ug/ul HA-Thy-1	3.2 μl (5 μg)
	water	441 μl
	2X HeBS	500 μl

於第 20 小時照相，曝光時間爲 1 / 2.5 秒

Restriction analysis of recombinant pAdEasy-Thy1following homologous recombination in *E. coli* BJ5183

The fragment of 4.5 Kb was generated when recombination took place between left arms, while 3.0 Kb one at the origins of replication

Western blot analysis of Thy-1 protein expression in the transfected cells



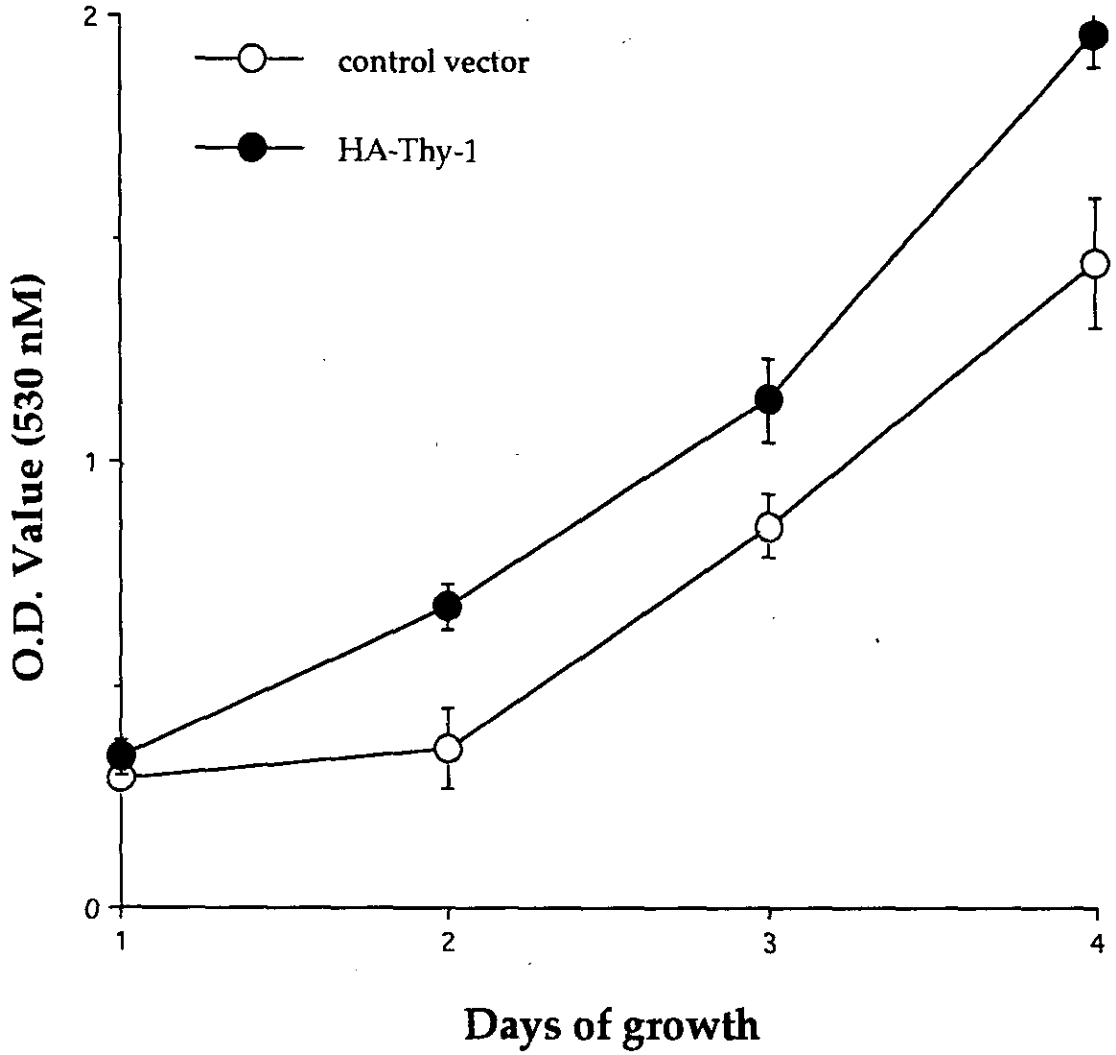


Figure 3. Transfection with human Thy-1 cDNA increased cell growth rate in the NIH3T3 cell measured with MTT assay