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Influences of wild- type p53 gene overexpression on the differentiation, apoptosis and expression of scavenger receptor CD36 in U937 cells*

YANG Xiang- dong[1], LIU Jun- wen[1], WANG Chun[1], LI Kai[2], TANG Wei- qing[3], LI Hong- xia[3], WANG Shu[3], LI Jian[3]

([1]*Institute of Cardiovascular Disease, Nanhua University, Hengyang* 421001, *China;* [2]*City of Hope National Medical Center, California, USA;* [3]*Department of Biochemistry, Beijing Institute of Geriatrics, Beijing Hospital, Beijing* 100730, *China*)

[ABSTRACT] **AIM:** To study the effect of wild- type p53 gene on the differentiation, apoptosis and expression of scavenger receptor CD36 in U937 cells. **METHODS:** Recombinant adenovirus vector with wild - type p53 gene was constructed and used to transfect U937 cells. With the expression of wild- type p53 gene following adenoviral infection, transfected U937 cells were largely promoted to differentiate into macrophages. **RESUITS:** Trypanblue- staining test demonstrated that the percentage of positive cells increased from (14.2 ±5.5)% to (64.6 ±9.2)% and nitroblue tetrazolium (NBT) reduction test reached similar results (6.3 ± 1.8)% *vs* (49.7 ±12.6)%. Furthermore, CD36 mRNA was up- regulated as confirmed by RT- PCR. The increased expression level of CD 36 was also detected by flow cytometry analysis. **CONCLUSION:** These results suggest that wild- type p53 gene can affect U937 cells differentiation and apoptosis, up- regulate expression of scavenger receptor CD36. It may have a potential significance on atherogenesis.

[KEY WORDS] Monocytes; Apoptosis; Antigens, CD36; Genes, p53; U937 cells

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The monocyte adhesion to the endothelium, then migration into the subendothelial space and differentiation into macrophages, are the key processes of atherosclerosis. Infiltrated macrophages carrying oxidized low density lipoprotein (ox- LDL) transforms into foam cells which are the main cell components at the atherosclerotic lesion. Apoptosis of foam cells plays an important role in the atherogenesis and its relevant complications. The relationship of apoptosis with plaque stability has been a highlight of this field. CD36 is one of the class B scavenger receptors, a multifunctional membrane receptor. CD36 mediates cell adherence, differentiation, and transmembrane signal transduction[1,2]. Other factors such as GM- CSF could induce the differentiation of U937 cells into macrophages, a process in which p53 gene acts as a regulator[3,4]. CD36 and p53 gene are up- regulated in the process of U937 cells foaming. Moreover, 40% of the uptake of ox- LDL is mediated by CD36[5- 7]. To investigate the influences of wild- type p53 gene on the expression of CD36 on U937 cells and its potential relationship with cellular differentiation and apoptosis, we constructed an adenovirus vector with wild - type p53 gene and transfected it into U937 cells. The modulation mechanism of the monocytes differentiation and CD36 expression has important significance in preventing the occurrence and progression in atherosclerosis.

MATERIALS AND METHODS

1 Construction of recombinant wild- type p53 gene adenovirus vector, AdCMV- p53

AdCMV - p53 was constructed as previously described[8]. Briefly, human wild- type p53 cDNA fragment

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Corresponding author Tel: 0086- 10- 65130302; E- mail: lijliy@hotmail.com

encoding the full- length P53 protein was first inserted into the plasmid vector pRC/CMV (Invitrogen). The wild-type p53 minigene cassette from the pRC/CMV plasmid vector containing the human cytomegalovirus promoter (CMV), the p53 cDNA fragment, and the polyadenylation site of the rabbit β- globin gene was then recovered and transferred into the *BamH* Ⅰ site of the shuttle plasmid PXCJL- 1. The resultant recombinant shuttle plasmid PX-CJLp53 was further co- transfected with the PJM17 plasmid into 293 cells using lipofectin reagent (Life Technologies, Inc.). Recombinant viruses were isolated by screening adenovirus plaques from 293 cell monolayers, and further characterized by restriction enzyme digestion mapping and by P53 protein expression in virus- infected p53-defective tumor cells. High- titer stocks of AdCMVp53 virus were obtained by CsCl ultracentrifugation preparation. Our previous sutdies show that transfection of Ad-CMV- lacZ vector has no significant effect on cells function[8, 12], so this experiment was conducted without Ad-CMV- lacZ vector group.

2 U937 cells culture and treatment

U937 cells were purchased from Shanghai Institute of Cell Biology of Chinese Academy of Sciences. Cells were grown in RPMI1640 (Gibco) medium containing 10% fetal serum (Gibco) at 37 ℃ in 5% CO_2. Cells were grown at a density of 2×10^8/L. Treatment group medium was replaced by culture medium plus AdCMV- p53 (10^{13}pfu/L). The efficiency of transfection was greater than 90 percent.

3 Cell cycle analysis with flow cytometry

After transfection (24 h or 48 h), the cells were washed twice with phosphate- buffered saline (PBS), fixed with 70% cold ethanol at 4 ℃ overnight. Resuspended cells were incubated in 50 mg/L RNase A for 1 h at 37 ℃, then suspended in 50 mg/L propidium iodide (PI) for 1 h at 4 ℃ before flow cytometry assay. Untreated cells were used as control. About 10 000 cells were counted in each tube.

4 Trypanblue- staining and nitroblue tetrazolium (NBT) reduction test

Trypanblue- staining test counted the living cells in per milliliter cells suspended solution, and NBT reduction test was carried out as described previously by Kamijo et al[9]. Mixed 0.1 mL NBT solution (1 g/L) with same volume of culture medium at 37 ℃ for 10 min, then suspended the cells, dripped 50 μL cells solution on the carryslip, after air- drying the carryslip, incubated with Wright staining solution for 30 min, washed the staining solution gently, counted the positive cells containing formazan by microscope.

5 Reverse transcription- polymerase chain reaction to detect the expression of CD36 mRNA

The total RNA was extracted for reverse transcripted. Reverse transcription (RT) was conducted according to the protocol of RT kit (Promega). GAPDH or β- actin used as control, GAPDH sense (5' - TGA AGG TCG GAG TCA ACG GAT TTT- 3'), antisense (5' - CAT GTG GGC CAT GAG GTC CAC CAC- 3'), 983 bp; β- actin sense (5' - GTG GGG CGC CCC AGG CAC CA- 3'), antisense (5' - CTC CTT AAT GTC ACG CAC GAT TTC - 3'), 540 bp; p53 sense (5' - CTA ACC GCG GTC CCT TCC CAG AAA ACC- 3'), antisense (5' - TAC AGT CAG AGC CAA CCT CAG GCG- 3'), 409 bp; CD36 sense (5' - GAG AAC TGT TAT GGG GCT AT- 3'), antisense (5' - TTC AAC TGG AGA GGC AAA GG - 3'), 389 bp. PCR was performed at 94 ℃ for 5 min at the first phase, then performed for 30 cycles of denaturation (94 ℃, 1 min), annealing (58 ℃, 1 min), and polymerization (72 ℃, 1 min), the last phase was performed at 72 ℃ for 10 min. Samples were analyzed by ethidium bromide staining on a 1.2% agarose gel.

6 Expression of CD36 receptor measured by flow cytometry

Cells harvested at 0 h, 12 h, 24 h, 36 h were washed with PBS, fixed in 4% polyformaldehyde at room temperature for 40 min. Cells were resuspended in PBS containing 5% human serum, incubated on ice for 10 min, centrifuged for 5 min at 115 g (800 r/min), incubated with 200 μL PBS containing 0.5% BSA and saturated dose of fluorescein isothiocyanate- conjugated (FITC) anti- CD36 monoclonal antibody on ice for 30 min plus 200 μL 1% polyformaldehyde. Flow cytometry was employed to the fluorescence value and 10 000 cells were counted in each tube.

7 Immunofluorescence assay (IFA) to detect the expression of CD36 on transfected U937 cells

Cells treatment was as described previously[6]. Cells were harvested after treatment and stained with FITC- anti - CD36 monoclonal antibody at 4 ℃ for 1 h under dark

chamber. Cells were observed by fluorescence microscope.

8 Statistical analysis

Results are presented as $\bar{x} \pm s$ from at least five separate experiments. Significance of differentiation on transfected U937 cells was assessed with the use of Student' s t test.

RESULTS

1 Detection of the efficiency of gene transfection

After transfection, cells were harvested at 0 h, 12 h, 24 h, 36 h, DNA was extracted and PCR was performed to determine the transfection efficiency of AdCMV – p53. Because p53 primer sequences were designed specifically according to p53 cDNA sequence, spanning one intron, so p53 gene could be obtained by PCR using cellular DNA as template just in the transfected cells. As shown by agrose gel electrophoresis (Fig 1), human wild– type p53 cDNA presents in U937 cells transfected with AdCMVp53. A 409bp product of PCR was not obtained in untransfected U937 cells.

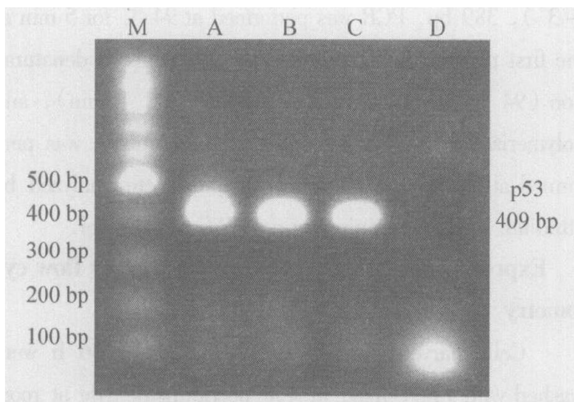

Fig 1 Detection of transfected p53 in U937 cells by PCR. M: 100 bp marker; Lane A: transfection for 36 h; Lane B: transfection for 24 h; Lane C: transfection for 12 h; Lane D: control (without transfection).

2 Cell cycle and apoptosis ratio analysis of transfected U937 cells

To investigate the effects on cell cycle of transfected cells, transfected cells were harvested at 12 h, 24 h, 36 h. Cell cycle was inhibited with most of the cells arrested in G_1 phase. Accordingly, cells at G_2/M and S phase were decreased remarkably (Tab 1). Figure of cell apoptosis analysis by flow cytometry was omitted.

3 Differentiation of transfected U937 cells

After tansfection for 12 h, cells were counted every 6 h. Cells of treatment group developed more slowly than those of control group. Trypanblue– staining showed that the positive cells of transfected cells increased, phagocytosis enhanced. NBT reduction test showed that the positive cells of transfected cells increased (Tab 2).

Tab 1 Analysis of U937 cell cycle and apoptosis ratio after transfection with AdCMV– p53

	U937 cells	AdCMV– p53 transfected cells(%)		
		12 h	24 h	36 h
G_1 phase	54.5	63.2	78.1	84.7
S phase	36.4	33.1	20.1	14.5
G_2/M phase	9.1	3.7	1.8	0.8
Apoptosis ratio	1.2	3.2	6.8	18.2

Tab 2 The effect of AdCMV– p53 transfection on differentiation of U937 cells

Group	Percentage of positive cells(%)	
(n= 5)	Phagocytosis	NBT reduction
Control	14.2 ±5.5	6.3 ±1.8
Experiment	64.6 ±9.2*	49.7 ±12.6*

Results are presented as $\bar{x} \pm s$. * $P < 0.05$ vs control.

4 Expression of CD36 mRNA detected by RT– PCR

CD36 gene level in U937 cells was detected by RT– PCR. A time dependent increase in CD36 expression was observed after transfection. Results of RT – PCR showed that CD36 mRNA level was up– regulated after U937 cells were transfected with AdCMV– p53 (Fig 2).

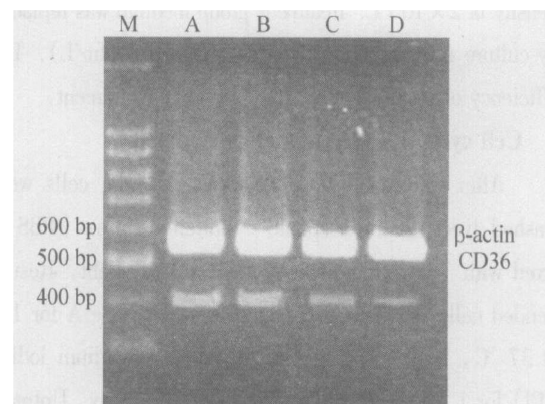

Fig 2 Expression of CD36 mRNA in U937 cells transfected with AdCMV– p53 by RT– PCR. Results are from one representative experiment. M: 100 bp marker; A: transfection for 36 h; B: transfection for 24 h; C: transfection for 12 h; D: control.

5 Expression of CD36 receptor detected by flow cytometry

Flow cytometry analysis results of cells with FITC– anti– CD36 monoclonal antibody showed that the average

fluorescence value of transfected cells (Red) increased significantly and the highest peak moved rightward. Twelve - hours after transfection, expression of CD36 increased mildly, but the expression increased obviously after transfection for 24 h and 36 h. These results were consistent with RT- PCR (Fig 3) .

6 Expression of CD36 receptor detected by IFA

Cells labeled by FITC- anti- CD36 monoclonal antibody were photographed by fluorescence microscope in order to observe the expression of CD36. Similar to flow cytometry results, the FITC fluorescence of transfected cells enhanced in a time- dependent manner (Fig 4) .

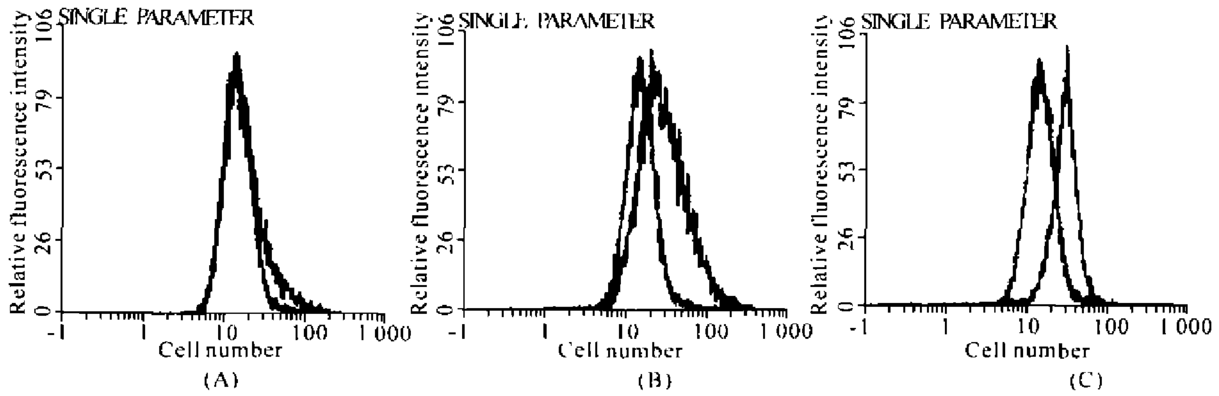

Fig 3 Expression of CD36 receptor on U937 cells after transfection with AdCMV- p53 was detected by flow cytometry analysis (10 000 cells/ sample, *n*= 5) . Results are from one representative experiment. A: transfection for 12 h; B: transfection for 24 h; C: transfection for 36 h.

Fig 4 Expression of CD36 receptor on U937 cells after transfection with AdCMV-p53 was detected by immunostaining with the FITC-CD36 antibody (FITC-immunostaining, ×400).
A: control; B: transfection for 12 h; C: transfection for 24 h; D: transfection for 36 h.

DISCUSSION

The monocytes migrate into the subendothelial space and become activated under the influence of local chemokines such as monocyte chemoattractant proteins- 1 (MCP- 1). These migrated and activated monocytes can further differentiate into macrophages and express certain crucial scavenger receptors required for the ingestion of modified lipids, by which they become macrophage foam cells. It is well known that the predominant role of the macrophage in atherosclerosis is to ingest and dispose of atherogenic lipids. Additionally, activated macrophages also express a variety of proinflammatory cytokines and growth factors that contribute to the development of the plaque. It is suggested that activated macrophages can induce vascular smooth muscle cells (VSMCs) apoptosis by direct cell- cell contact. If the lesion ruptures or erodes platelets rapidly accumulate, and intravascular thrombosis occurs. This leads to the acute coronary syndromes of unstable angina or even myocardial infarction. Plaques with a large lipid pool and a thin fibrous cap are more prone to rupture than those with a thick cap, partly because a thick fibrous cap is more resistant to local mechanical stresses. However, the most important determinant of plaque stability is the composition of the fibrous cap. Inflammatory cells including macrophage foam cells and a relative paucity of VSMCs leads to plaque rupture. Elucidation of mechanism of monocytes differentiation and macrophage foam cells formation/ apoptosis may provide an important research and therapeutic target for preventing atherogenesis[10, 11].

Wu et al[3, 4] demonstrated that GM- CSF, phorbol 12- myristate 13- acetate (PMA) and interferon- γ (IFN- γ) induced the differentiation of U937 cells. The p53 gene plays an important role in the process of differentiation induced by GM- CSF. We recently found that ox - LDL could induce the differentiation of U937 cells and up- regulate the expression of scavenger receptor CD36, p53 gene and p21 gene in the experiment of U937 cells foaming and apoptosis induced by oxLDL. Tang et al[12] also found that wild- type p53 gene transfected effectively c- jun and p21, with more apoptosis observed. To further study the effects of wild- type p53 gene on monocytes differentiation and apoptosis, U937 cells were transfected with AdCMV- p53. The results showed that overexpression of wild- type p53 gene could induce cells differentiation. U937 cells growth became slower time- dependent, and the cell cycle was arrested at G_1 maybe due to the proposed dual role of p21 in regulating G_1 and early S- phase events through its binding to CDKs and/ or proliferating cell nuclear antigen (PCNA)[13]. Moore et al[14] demonstrated that in microglia and other tissue macrophages, beta- amyloid initiates a CD36- dependent signaling cascade involving the Src kinase family members, Lyn and Fyn, and the mitogen- activated protein kinase, p44/ 42. The finding that engagement of CD36 by beta- amyloid initiates a Src kinase- dependent production of inflammatory mediators in cells of the macrophage lineage reveals a novel receptor- mediated pro- inflammatory signaling pathway of potential therapeutic importance. Furthermore, thrombospondin (TSP- 1) binding to CD36 could activate Fyn/ Src kinase signaling to cause apoptosis of endothelial cells. Wintergerst et al[15] identified that binding of ox- LDL to CD36 initiates a yet unkown oxLDL- specific signaling event, with a rapid activation of caspase- 3 resulting in apoptosis of human macrophages. Our experiments suggest that transfection of wild- type p53 gene could up - regulate expression of CD36, a mechanism that may be associated with the Fyn/ Src kinase signal and the caspase - 3 signal. It is a novel role for CD36 in macrophage biology for the development of atherosclerotic lesions. Further studies are required to elucidate the underlying mechanism.

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([1]Institute of Cardiovascular Disease, Nanhua University, Hengyang 421001, China; [2]City of Hope National Medical Center, California, USA; [3]Department of Biochemistry, Beijing Institute of Geriatrics, Beijing Hospital, Beijing 100730, China)

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野生型 p53 基因导入对 U937 细胞分化、凋亡和 CD36 受体表达的影响

杨向东[1], 刘俊文[1], 王 春[1], 李 凯[2], 唐蔚青[3], 李红霞[3], 王 抒[3], 黎 健[3]

([1] 南华大学心血管病研究所, 湖南 衡阳 421001; [2]City of Hope National Medical Center, California, USA;
[3]卫生部北京老年医学研究所生化室, 北京 100730)

[摘 要] 目的: 研究野生型 p53 基因重组腺病毒载体(AdCMV- p53)导入对 U937 细胞分化、凋亡和清道夫受体 CD36 表达的影响。方法: AdCMV- p53 导入 U937 细胞后,用细胞计数、细胞周期分析、台盼蓝染色排除法计数细胞悬液中的活细胞数目和 NBT 还原反应观察其对 U937 细胞生长、分化的影响; RT- PCR、免疫荧光和流式细胞分析检测 AdCMV- p53 导入对 CD36 表达的影响。结果: AdCMV- p53 可以高效导入 U937 细胞,野生型 p53 基因导入促进 U937 细胞向巨噬细胞分化,台盼蓝染色发现实验组阳性细胞数(64.6 ±9.2)% 较对照组(14.2 ±5.5)% 明显增多,吞噬能力增强; NBT 还原反应实验组(49.7 ±12.6)% 较对照组(6.3 ±1.8)% 升高。RT- PCR 和流式细胞分析检测,野生型 p53 基因导入使得 CD36 mRNA 转录增强, CD36 蛋白表达增加。结论: 野生型 p53 基因能影响细胞分化和凋亡,并上调清道夫受体 CD36 的表达,对于动脉粥样硬化的预防和基因治疗具有潜在意义。

[关键词] 单核细胞; 细胞凋亡; 抗原, CD36; 基因, p53; U937 细胞

[中图分类号] R363 [文献标识码] A