SUMO-1 Enhancing the p53-induced HepG2 Cell Apoptosis

LU Xingrong (卢星榕), YI Jilin (易继林)*
Department of General Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Summary: In order to investigate the effect of small ubiquitin-like modifier-1 (SUMO-1) on the p53-induced HepG2 cell apoptosis, HepG2 cells were transfected by recombinant plasmids as pwtp53, pMDM2 and pSUMO-1 respectively. Western blot was employed to detect the protein expression of the transfected recombinant plasmids and the rate of apoptosis was measured by flow cytometry. The results showed that in cells transfected with pwtp53 and pwtp53+pSUMO-1, the apoptosis rate was (16.79±1.62) % and (18.15±1.36) % respectively, while transfected with pwtp53+pMDM2, the rate was decreased to (5.17±1.23) %. The apoptosis rate was (14.06±1.84) % in the cells transfected with pwtp53+pMDM2+pSUMO-1, significantly higher than that in the cells Transfected with pwtp53+pMDM2 ($P<0.01$). The apoptosis rates in the cells were all less than 2 % and had no significant difference among the groups. It was suggested that in the HepG2 cells, SUMO-1 can increase the apoptosis induced by wild-type p53 through binding to p53 protein, post-translational modification and inhibiting the p53 degradation by MDM2.

Key words: small ubiquitin-like modifier-1; p53 gene; murine double minute gene 2; HepG2 cell; transfection; apoptosis

Wild-type p53 gene (wtp53) can induce cell apoptosis and inhibit oncogenesis[1,2]. The protein of muring double minute gene 2 (MDM2) was induced by wtp53. After MDM2's binding to p53 protein, it can stimulate the p53 degradation through the ubiquitin pathway, forming the p53-MDM2 negative feedback circle[3,4]. Small ubiquitin-like modifier-1 (SUMO-1) plays an crucial role in the protein post-translational modification[5,6]. SUMO-1 can compete the receptor binding sites of some proteins with ubiquitin and protect substrates such as p53 protein from being degraded by ubiquitin[7], then enhance the stability of tumor suppressor and their transcriptional activities[8]. In this experiment, the HepG2 cells were transfected by pwtp53, pMDM2 and pSUMO-1 in order to observe the effects of SUMO-1 on the HepG2 cell apoptosis induced by wild-type p53.

1 MATERIALS AND METHODS

1.1 Cell Line

The human hepatoblastoma cell line (HepG2) was grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS), 2 mmol/L glutamine, 100 U/ml penicilin and 100 μg/ml streptomycin before transfection (Hyclone).

1.2 Transient Transfection of Plasmids

HepG2 cells were incubated in a 6-well plate which contained 5×10^5 cells/well and cultured to reach 80 %—90 % cell confluence. Cells were then transfected with pcDNA3-wtp53 (pwtp53), pCMV-HDM1B (pMDM2), pcDNA3-His_6-SUMO-1 (pSUMO-1) and empty vector according to the protocol of Lipofectamine2000 reagent (Invitrogen, USA). The different transfected cell lines were harvested after further 36 h incubation. All of above plasmids were generous gifts of Prof. Dimitris Xirodimas (St. Andrews College, England).

1.3 Western Blotting

For the cells without pSUMO-1 transfection, the protein was extracted by the routine method[9]. As for the groups transfected with pSUMO-1, protein lysates containing the following buffers was applied 2 ml per well to prevent the degradation of SUMO-1 protein: 6 mol/L guanidinium-HCl, 0.1 mol/L Na_2HPO_4/NaH_2PO_4, 0.01 mol/L Tris/HCl (pH 8.0), 5 mmol/L imidazole and 10 mmol/L β-mercaptoethanol. The lysates were then purified by Ni^{2+}-NTA-agarose beads (Qiagen, Germany) and the eluates were dissolved in 200 μl 5 % SDS plus the following buffers: 0.15 mol/L Tris - HCl (pH 6.7), 200 mmol/L imidazole, 0.72 mol/L β-mercaptoethanol and 30 % glycerol. The eluate protein concentration was measured by Coomassie brilliant blue method. The eluates were separated by 10 % SDS-PAGE, transferred to a nitrocellulose filter. 5 % defatted milk powder was added to block nonspecific binding site over night. Subsequently, anti-p53 (Santa Cruz, USA), anti-MDM2 (Biosource, USA) and anti-SUMO-1 (Santa Cruz, USA) monoclonal antibody were added for incubating 12 h and horseradish peroxidase labelled second antibody for incubating 2 h at room temperature. After addition of the 3,3'-diaminobenzidine (DAB) substrate, the photographs of specific protein bands which had been shown were

LU Xingrong, male, born in 1970, Doctor in Charge M.D., Ph.D.
* Corresponding author

taken.

1.4 Measurement of Cell Apoptosis Rate

After trypsinization, centrifugalization and collection, PBS washing, 70 % ethanol fixation over night, PBS washing again and resolvation, adding RNase and PI (50 μg/ml), then staining away from light for 30 min, the apoptosis rate of the transfected cells was measured by flow cytometry (FACScan, USA). The cells without any transfection were taken as controls. Each group was repeatedly measured by 3 times and each sample included 1×10^4 cells.

1.5 Statistical Analysis

The experiment data were presented in $\bar{x}\pm s$. Differences in proportions were evaluated by the t-test which check standard was $P<0.01$.

2 RESULTS

2.1 Protein Expression of Exogenous Genes in HepG2 cells after Transfection

Western blotting revealed that the cells transfected with the pwtp53 expression plasmid had a band of 53 kD (1 kD = 0.992 : 1 ku) (fig. 1A, lanes 3, 6, 7 and 9, indicating p53 protein). Cells co-transfected with the pSUMO-1 and pwtp53 had a higher band of 65 kd (fig. 1B, lanes 7 and 9, indicating SUMO-1 covalently linked to p53 protein). The cells transfected with the pMDM2 had a band of 90 kd (fig. 1C, lanes 4, 6, 8 and 9, indicating MDM2 protein). Similarly, the cells co-transfected with the pSUMO-1 and pMDM2 also had a higher band of 110 kd (fig. 1D, lanes 8 and 9, indicating SUMO-1 linked to MDM2 protein). Merely the trace of p53 protein and no MDM2 protein was detected in cells not transfected with any plasmid or only transfected with empty vector pcDNA3 and pSUMO-1 (fig. 1A, lanes 1, 2, 4, 5 and 8; fig. 1C, lanes 1, 2, 3, 5 and 7). The cells transfected with the pSUMO-1 had a band of 11 kd (fig. 1E, lanes 5, 7, 8 and 9, indicating SUMO-1 protein).

2.2 Apoptosis Rate of HepG2 Cells after Transfection

In the cells transfected with pwtp53 and pwtp53 + pSUMO-1, the apoptosis rate was (16.79±1.62) % and (18.15±1.36) % respectively (fig. 2, A and B); When the cells were transfected with pwtp53 + pMDM2, the apoptosis rate was decreased to (5.17±1.23) % (fig. 2, F). The apoptosis rate was increased to (14.06±1.84) % (fig. 2, B) after the cells were transfected with pwtp53 + pMDM2 + pSUMO-1 as compared with the cells transfected with pwtp53 + pMDM2 ($P<0.01$). The apoptosis rate in the null cells and the cells transfected with pMDM2, pSUMO-1, pMDM2 + pSUMO-1 and pcDNA3 plasmid was (1.63±0.57) %, (1.80±0.64) %, (1.96±0.61) %, (1.19±0.22) % and (1.04±0.38) % recpectively, all less than 2 % and had no significant difference among groups (fig. 2, A, B, D, E, H).

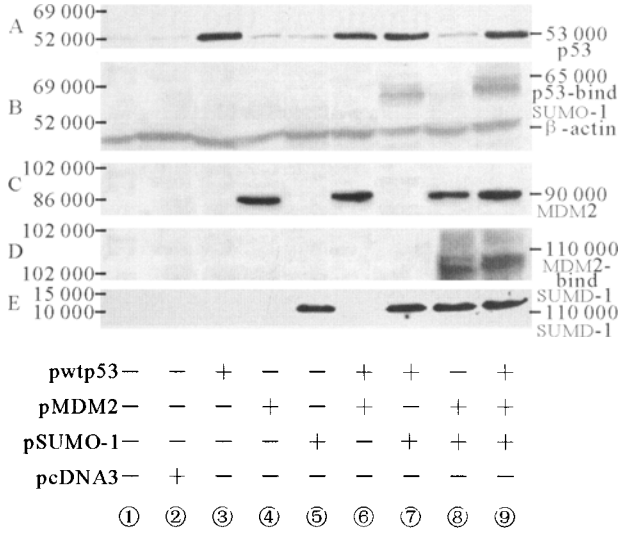

Fig. 1 Protein expression of exogenous genes in HepG2 cells after transfection

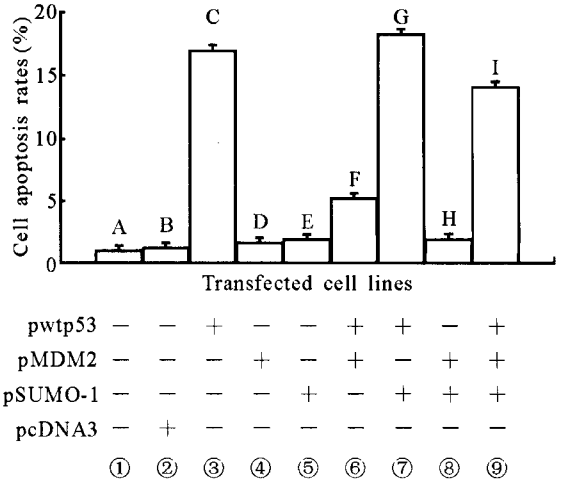

Fig. 2 The apoptosis rate of HepG2 cells after transfection
C, G and I vs F, $P<0.01$

3 DISCUSSION

The wild-type p53 is a principal mediator of growth arrest in G_1 phase, senescence, and apoptosis in response to a broad array of cellular damage. As cells that have mutant-type p53 or loss of p53 function are unable to respond appropriately to stress, they make uncontrolled growth of cells that favor the development of malignant tumor[1]. Transfecting the p53 plasmid into tumor cells would theoretically restore p53 gene function and its program death signal to induce apoptosis. This ideal has been proved in some tumor therapies such as medulloblastoma, mammary cancer and carcinoma of the head and neck[2]. The results in this study indicated that the cells without any transfection or transfected with empty vector only had the trace of p53 expression. However, the cells trans-

fected with pwtp53 had a higher p53 level and its apoptosis rate was (16.79±1.62) %, significantly higher than in the non-transfected cells ($P<0.01$). So recovering the expression of wtp53 gene would play a critical role in tumor therapy.

The oncogene MDM2 encodes a kind of zinc-finger protein. MDM2 is known as a member of ubiquitin family and a ubiquitin ligase E3 for tumor suppressor p53[3]. An important function of MDM2 is to bind to p53 protein, inhibiting the function of the latter as a transcription factor and stimulating p53 degradation through the ubquitin pathway. p53 also activates MDM2 expression at the level of transcription, suggesting that MDM2 can act as a negative feedback regulator of p53[4]. In order to investigate the effects of SUMO-1 on the apoptosis induced by wtp53 gene in HepG2 cells, we introduced the pCMV-HDM1B expression plasmid (pMDM2) which included the human homologue of the MDM2 gene. It was shown that the cells transfected with the pMDM2 had a protein band of 90 kD. The cells co-transfected with pwtp53 and pMDM2 had a lower apoptosis rate than that of the cells only transfected with pwtp53 ($P<0.01$), demonstrating that MDM2 could degradee p53 and indeed abrogate the ability of p53 to induce cell apoptosis. MDM2 protein expression clould not be detected in cells not transfected with pMDM2. So in normal condition, HepG2 cell itself does not have or have very less MDM2 expression.

Over half of all human tumours contain mutations p53 gene that functions abnormally. However, in tumours that have no mutated p53 gene, the over-expression of MDM2 protein is often present[10]. So, how to restore the anti-tumor function of p53 and weaken the degradation of p53 by MDM2 is the key problem in tumor therapy. Among the post-translational modification systems, studies about SUMO-1 have become a hot point succeeded to ubiquitin. In mammalian cells, the majority of SUMO-1 exists in the conjugated form and its monoform's molecular weight is about 11 kD, whereas the SUMO-2 and SUMO-3 isoforms exist primarily as free proteins that are subject to rapid conjugation after cellular stress and educe their special functions[5]. Some 60 SUMO target proteins have been reported so far, of which a significant number are either transcription factors or other proteins involved in regulating cell cycle such as p53 and MDM2[6]. SUMO-1 affects target proteins function by competing with ubiquitin for receptor site of substrate, preventing correlated proteins from ubquitin's degradation[7] and then increases the stability and transcriptional activity of suppressor genes such as p53[8]. This trial has the same result as above reports. Beside the p53 band (53 kD) and MDM2 band (90 kD), the cells transfected with pwtp53 + pMDM2 + pSUMO-1 had higher bands of SUMO-1-p53 (65 kD) and SUMO-1-MDM2 (110 kD) which had a gradient trail after the main band. This phenomenon would be considered as various number of SUMO-1 molecules combined to p53 or MDM2 and formed different molecular weight of protein complex. The apoptosis rate would up again to (14.06±1.84) % and the difference of rate was significant compared to the cells transfected with pwtp53 + pMDM2 ($P<0.01$). The results illustrated that SUMO-1 could increase the apoptosis of HepG2 cells induced by wild-type p53 through combining to p53 protein, post-translational modification and inhibiting p53 degradation by MDM2.

REFERENCES

1 Ko L J, Prives C. p53: puzzle and paradigm. Genes Dev, 1996,10(9):1054

2 Rosenfeld M R, Meneses P, Dalmau J *et al*. Gene transfer of wild-type p53 results in restoration of tumor-suppressor function in a medulloblastoma cell line. Neurology, 1995,45(8):1533

3 Honda R, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett, 1997,420(1):25

4 Haupt Y, Maya R, Kazaz A *et al*. Mdm2 promotes the rapid degradation of p53. Nature, 1997,387(6630):296

5 Saitoh H, Hinchey J Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J Biol Chem, 2000,275(9):6252

6 Muller S, Hoege C, Pyrowolakis G *et al*. SUMO, ubiquitin's mysterious cousin. Nat Rev Mol Cell Biol, 2001,2(3):202

7 Melchior F. SUMO-nonclassical ubiquitin. Annu Rev Cell Dev Biol, 2000,16:591

8 Rodriguez M S, Desterro J M, Lain S *et al*. SUMO-1 modification activates the transcriptional response of p53. Embo J, 1999,18(22):6455

9 Sambrook J, Ftitsch E, Mauitis T. Molecular cloning. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1988.362—371, 792—793

10 Zheleva D I, Lane D P, Fischer P M. The p53-Mdm2 pathway: targets for the development of new anticancer therapeutics. Mini Rev Med Chem, 2003,3(3):257

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