Proceedings of the Korean Nuclear Society Spring Meeting

Gyeongju, Korea, 2004

ROS Mediated Enhancement of Radiation Response by Sps

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Abstract

We employed subtractive hybridization methods to identify radiation induced genes. These genes are expected to have some role in radiation response. among the identified genes, sps (interchangeable with HSDP) was shown to modulate radiation response. Stable cell line which overexpress sps enhanced p53 activity by increasing protein stability and activity as a transcription factor. Increased p53 was correlated with increased phosphorylation of serine residue 15 of p53 protein. sps cell lines produced more p21 and MDM2 which are two representative p53 target genes. Increased level of p53 was due to increased protein stability.

Half life of p53 was increased in sps cell lines. Increased P53 level was correlated with enhanced cell killing by irradiation in sps cell lines. Both UV and gamma radiation caused lowered cell viability of sps cell line. We conclude that overexpression of sps caused upregulation of p53, a main mediator of radiation response, and sensitized cells that stably expressed sps.

Introduction

Compex molecular systems have evolved in response to stress conditions including heat, nutritional deprivation, virus infection and irradiation. Cellular responses to irradiation are especially interesting in that it involves damage to genetic material. DNA damage caused by radiation is sensed and the signal is transduced and repair machinery is mobilized to minimize the damage. In the process, p53 protein is involved in DNA repair and cell cycle or cell death. To prevent amplification of damaged DNA and to facilitate repairing process, cell cycle is arrested by a checkpoint system. Once the damage is reaches over the limit, cell commits death to contain the damage within the cell. We were interested in radiation response, especially in molecular level. Irradiation of cells with ionizing radiation (IR) causes differential gene expression. Up- or down-regulation of various genes can be monitored by using microarray or by similar approach. We used subtractive hybridization to select genes that are able to modulate radiation response. Here we report one of the gene sps(HSDP).

Material and methods

Subtractive hybridization: In order to perform subtractive hybridization, total RNA and mRNA were isolated by using Trizol TM (Life technology, MD, USA) and oligotex bead (Qiagen Co., CA, USA) respectively. Kit for subtractive hybridization was purchased from Clontech and its instruction manual was followed. Briefly, isolated mRNA was used as a template for synthesis of first and second strand cDNA. Longer cDNA was digested with Rsa1 to ligate two different adaptors by which identification of differentially expressed cDNA is facilitated. First and second hybridization were 8 hours and 16 hours respectively. Screened cDNA were ligated into T-easy vector and used to transform competent bacteria. Transformed bacteria were screened for their inclusion of cDNA fragment by blue and white color selection. White colonies that indicate the presence of cDNA insert were screened further for their upregulation by differential screening. Colonies selected after two rounds of screening were processed for sequencing analysis.

RT-PCR analysis: Screened cDNAs from subtractive hybridization analysis were examined for their differential expression by RT-PCR. Most PCR reactions produced less than 0.5kb products by using the following cycle condition. 94 degree 3min.-->15 to 35 cycles of (94 degree 30 sec.-->56 degree 30sec.-->72 degree 35 sec.). PCR products were run in agarose gel electrophoresis.

Clonogenic assay and western analysis:

Clonogenic assay was performed to determine the effect of HSDP on cell viability. 500-1000 cells were plated and treated as indicated then surviving cells were detected by their colonies. Western analysis was used to determine protein levels. Specific antibodies were used as indicated in each figure legends.

Results and discussion

In order to elucidate the function of HSDP, we produced stable cell lines that expressed HSDP (Fig. 1). Since the antibody to HSDP was not available, we added flag tag to amino terminus of HSDP protein. All HSDP detection were done with FLAG antibody. Out of 12 cell lines whose HSDP expression were variable, we selected two highest expressing cell lines #5 and #6. FLAG tagged fusion proteins were selectively detected in the stable cell line but not in controls. Expression of control protein actin was not variable.

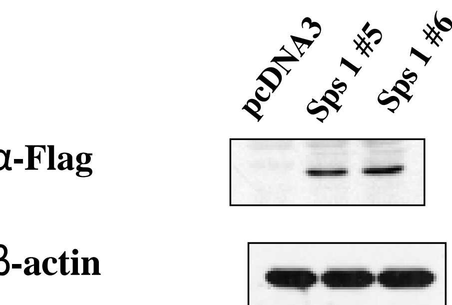
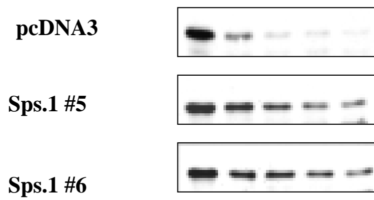


Figure 1. Western analysis of HSDP stable cell lines. FLAg-HSDP fusion proteins were detected with FLAG antibody. Actin is endogenous control and shows no change by HSDP expression.

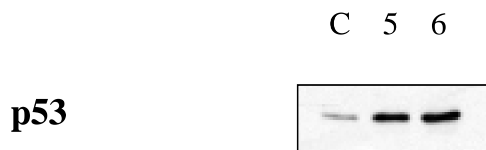
With the #5 and 6 HSDP cell lines, we examined p53 and p53 target genes. Since p53 is a main regulator of radiation response, modulation of p53 in terms of protein level or activity could have impact on radiation response.

HSDP cell lines produced more p53 proteins compared to control (Fig. 2). Increased p53 in HSDP cells was phosphorylated on its serine 15 residue. Phosphorylation of this site has been associated with increased protein stability. To confirm the fact, we examined time course of p53 degradation to determine half-life of p53 in each cell lines. Half-life of p53 was longer in HSDP cell lines indicating that increased p53 level was caused by increased half-life (Figure 2). Incresed p53 protein was correlated with MDM2 protein which is one of the targets of p53. This indicates that p53 proteins are functionally active. From the results, we could conclude that stable overexpression of HSDP caused increased p53 protein and activity by protein stabilization.

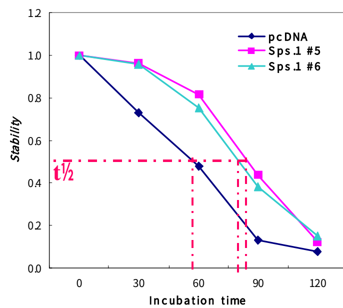
a. p53 Half-life



b. Western analysis of p53 in control (C), and #5, 6 sps cell lines



c. Graphical presentation of Half-life data.



d. Phosphorylation at 15 ser residue of p53.

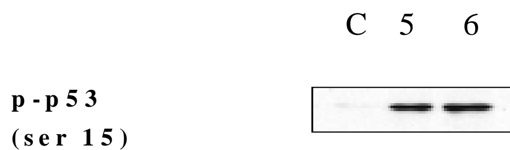
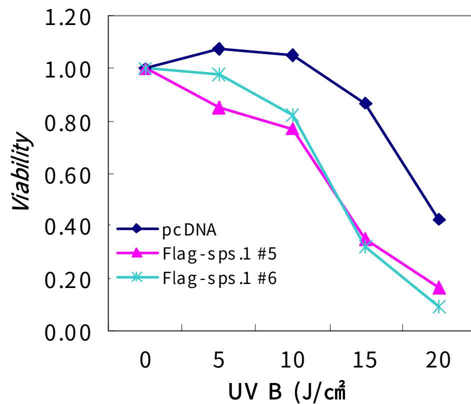


Figure 2. a, d: Western analysis of p53 and pp53. b, c: Haf-life determination of p53 by cycloheximide treament and western analysis of p53.

In order to elucidate the role HSDP played in radiation response, we irradiated cell lines with

ionizing and non-ionizing radiation. Irradiation of HSDP cell lines with UV-B caused enhanced cell killing compared to control (Figure 3).

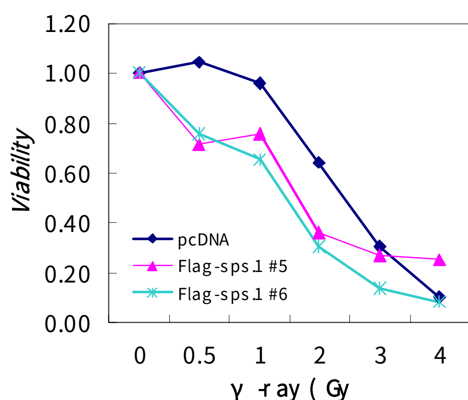


	pcDNA	Flag-sps.1 #5	Flag-sps.1 #6
0	1.00	1.00	1.00
5	1.08	0.85	0.97
10	1.05	0.77	0.82
15	0.87	0.35	0.32
20	0.42	0.17	0.09

Figure 3. UV-B irradiation and cell viability as determined by clonogenic assay.

Among the two HSDP cell lines, #5 had more cell killing. This effect on cell viability was correlated with p53 stability. P53 was more stable in HSDP #5 than in #6. Considering the effect of p53 on cell death, it is not that surprising that cells with more p53 produced more cell killing.

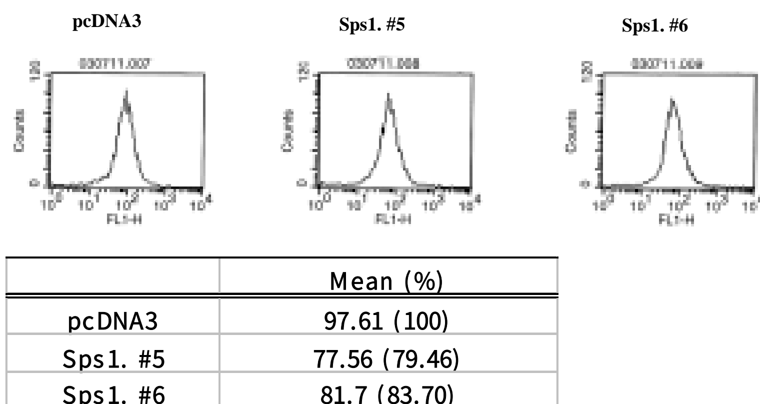
Unlike non-ionizing UV-B, ionizing radiation, gamma irradiation causes DNA breakage. Since p53 is also involved in DNA repair as well as cell death, we were intersted whether which effect of p53 is dominant in HSDP cell line. Gamma irradiation of HSDP cell line produced more cell killing or lowered cell viability compared to control (Figure 4).



	pcDNA	Flag-sps.1 #5	Flag-sps.1 #6
0	1.00	1.00	1.00
0.5	1.04	0.71	0.76
1	0.96	0.75	0.65
2	0.64	0.36	0.31
3	0.31	0.27	0.14
4	0.10	0.25	0.08

Figure 4. Gamma irradiation and cell viability as determined by clonogenic assay.

If p53 was the only determinant of cell fate, the results does not explain how contrasting effect of p53 is reconciled. In other words, p53 has both pro-survival and cell death-promoting activities. In HSDP cell lines, the balance was tilted toward cell death. Are there additional factors that favors cell death in HSDP cell lines when they are irradiated? To answer the question, we measured cellular ROS (reactive oxygen species) level (Figure 5).



	Mean (%)
pcDNA3	97.61 (100)
Sps1. #5	77.56 (79.46)
Sps1. #6	81.7 (83.70)

Figure 5. ROS levels determined by FACS analysis.

Considering the fact that increased ROS level favors cell proliferation, the correlation of severe reduction of ROS in HSDP cell lines with lowered cell viability by irradiation is interesting. This suggests that modulation of cellular ROS level can have an impact on radiation response by regulating p53's activity. Identification of genes that modulate p53 and their effect on radiation response should facilitate radiation based therapy.