Journal of Natural Science
Pai Chai University, Korea
Vol. 9, No. 1: 31~37, 1996

Lack of p53 Gene Nucleotide Change in Mutation Hot Spots During HeLa Cell Apoptosis by Adriamycin

Seung-Wook Ryu, Jung Woo Kim and Eun-Hee Kim
Division of Chemistry and Life Science, Pai-Chai University,
Daejon 302-735, Korea

아드리아마이신에 의한 HeLa 세포의 자살 과정 중 p53 유전자의 돌연변이 빈발 부위에서의 핵산 변화의 不在

류승욱, 김정우, 김은희
화학 및 생명 과학부, 배재대학교, 대전광역시 302-735, 한국

세포의 자살 현상은 항암제를 이용한 치료법에 있어 중요하다. p53 은 세포 자살을 유발하여 암세포를 죽이는데 핵심적 요소임이 밝혀졌다. 그러나 최근의 연구는 p53 과 무관한 세포 자살 경로가 있음을 보였다 (Gaftenhaus 외, 1996). 본 저자들은 유전독성적 항암제인 아드리아마이신에 의하여 유도된 세포 자살 과정에서 p53 유전자의 돌연변이가 일어나는지를 관찰하였다. 그러므로 본 연구는 아드리아마이신에 의하여 유도된 세포 자살 과정에서 HeLa 세포 집단에서의 p53 유전자의 돌연변이 상태를 조사하였다. DNA 분절 현상으로 관찰한 바, 본 실험 조건하에서는 아드리아마이신을 1 uM 농도로 12 시간 처리하였을 때 세포 자살 현상이 일어났다. p53 유전자의 돌연 변이 상태를 관찰하고자 돌연변이 빈발 부위가 존재한다고 알려진 exon 5, 7 및 8 부위를 중합 효소연쇄반응으로 증폭하여 핵산 서열 변화를 검색하였다. 그러나 세포 자살이 일어나고 있는 HeLa 세포 집단에서 핵산 변화는 관찰되지 않았다. 그러므로 본 연구는 p53 유전자 손상없이 아드리아마이신에 의하여 세포 자살이 유도됨을 밝혔다.

Apoptosis is an important event in the anticancer drug therapy. p53 was demonstrated to serve as a key component to lead tumor cell death by inducing apoptosis. However, recent study showed the presence of p53 independent apoptotic pathway (Gaftenhaus *et al.*, 1996). We were curious to know if apoptosis induced by adriamycin, a genotoxic anticancer agent, involved p53 gene mutation. Thus this study investigated the p53 gene mutation status among HeLa cell population during apoptosis induced by adriamycin. Under our experimental condition, 12 hour treatment of 1 μM adriamycin caused apoptosis which was monitored by DNA fragmentation assay. In order to see the p53 gene mutation status, exons of 5, 7 and 8 of p53 gene, where previously reported p53 mutation hot spots reside, were amplified by PCR and nucleotide sequence change was scanned. However, no nucleotide change was observed among apoptotic HeLa cell population. Therefore this study demonstrated that adriamycin induced apoptosis without causing p53 gene damage.

Key words : Apoptosis, Adriamycin, p53, HeLa, Mutation

I. INTRODUCTION

Adriamycin has been widely used as an effective anticancer agent. Although it remains as one of the most effective components of combination therapy for the treatment of a wide range of tumor, the mode of action of this drug has not yet been resolved. Some proposed modes of action involves DNA related damage to template function, impairment of topoisomerase II activity, membrane-related effect, or products of bioreductive activation (Cullinane *et al.*, 1994 ; Vichi *et al.*, 1992 ; Powis, 1987). However no single mechanism accounts for all of the effects associated with the chemotherapeutic use of adriamycin.

Meanwhile recent view that apoptosis is an important tumor cell killing mechanism by various anticancer drugs gains support (Lowe *et al.*, 1994). Thus studies to reveal molecular mechanism of apoptosis induction by adriamycin are active.

Tumor suppressor p53 have been suggested to be a key component to direct apoptosis (Enoch and Norbury, 1995 ; Lowe *et al.*, 1994 and 1993). The failure of apoptosis from p53 gene knockout mice again supports this view (Clarke *et al.*, 1993). But the presence p53 independent apoptotic pathway was reported i.e. glucocorticoids induced apoptosis without functional p53 in thymocytes (Clarke *et al.*, 1993). The story gets more complicated. Gaftenhause and colleagues (1996) induced apoptosis by DNA-damaging agent in some lymphocytes without functional p53. Thus a lot needs to be studied about the relationship between p53 status and apoptotic agents in various cell lines.

Most studies to date seems to focus whether the apoptosis occurs or not with pre-defined p53 gene status. However this might not represent the *in vivo* situation during anticancer chemotherapy. p53 gene status might change during drug's chemotherapeutic action.

Adriamycin is a genotoxic, DNA-damaging anticancer agent. The fact that more than 99.8% of adriamycin was bound to DNA in living cells and over 80% of administered adriamycin was found in association with DNAs in human tumor biopsies strongly supports this idea (Cullinane *et al.*, 1994). p53 mutation is the most frequently observed one in human cancer. Therefore there is a possibility that adriamycin might damage p53 gene itself during its chemotherapeutic action. Thus this study aimed to monitor the p53 gene status change among HeLa cell population during apoptosis by adriamycin treatment. This way the under standing of the relationship between p53 mutation and apoptosis by adriamycin could be deepened.

II. MATERIALS & METHODS

1. Chemicals

Adriamycin was purchased from IL Dong pharm. Co., Korea. Cell culture material was purchased from GIBCO/BRL, USA. 123 ladder DNA marker was purchased from Promega, USA. Sequenase version 2.0 Kit was purchased from U.S.B., USA. All other reagents were purchased from Sigma, USA.

2. Cells

Cervix epileloid carcinoma (HeLa, ATCC # 1958) cell line were purchased from ATCC, USA. Chronic myelogenous leukemia cell line K-562 was generously given from Dr. Kong-Joo Lee, college of pharmacy, Ewha Womans University. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum containing 1% antibiotic mixture (100 U/ml penicillin G, 100 U/ml streptomycin) at 37 ℃ under a humidified 5% CO_2 atmosphere.

3. DNA fragment assay

Cells in their exponential phase of growth (approximately 3.0×10^4 - 1.0×10^7 cell / 10

ml) were treated with various concentration (0.1 - 100 μM) of adriamycin for each time point (0 hr - 24 hr). The drug was diluted to final concentration from freshly prepared 1 M adriamycin stock in distilled water and administered to cells. Following incubation with adriamycin, cell monolayer was washed twice with cold PBS. Subsequently the cells were trypsinized, harvested by centrifugation for 5 min at 1,500 rpm and washed twice with ice-cold PBS. DNA preparation was performed according to published method with some modification (Skladanowski and Konopa, 1993 ; Kaufmann *et al.*, 1993 ; Miyashita and Reed., 1992 ; Barry *et al.*, 1990). Cells were lysed in 200 μl lysing buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 10 mM NaCl, 0.5% SDS and 0.5 mg/ml proteinase K. After 1 hr at 50℃, 5 μl of 1 mg/ml RNAse A was added to each sample and incubation at 50℃ was continued for 2 hr. Following incubation, the salt concentration (5 M NaCl) was raised to 1 M, and tubes shaken vigorously. Samples were centrifuged (30 min, 2,000 rpm) and supernatants were collected, 2.5 volume of ethanol (95%) was added and DNA was precipitated overnight at -20℃. The DNA pellet was resuspended in 20 μl of 10 mM Tris-HCl (pH 7.4), 15 mM NaCl, 1 mM EDTA. The DNAs were separated by electrophoresis in 1.5% agarose gel for 1 hr at 50 V with running buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8). DNA was visualized by ethidium bromide staining photographed under UV illumination.

4. PCR amplification

Genomic DNAs prepared as above were used as templates for PCR amplification. Primers were designed to amplify exon 5, 7 and 8 of p53 with EcoRI linker for subsequent cloning. Primer sequences are following.

for exon 5
5F :
5′ - GGAATTCCTCTTCCTGCTGTACTC - 3′ (24mer)
5R :
5′ - GGAATTCGCCCCAGCTGCTCACC - 3′ (23mer)
for exon 7
7F :
5′ - GGAATTCCTAGGTTGGCTCTGAC - 3′ (23mer)
7R :
5′ - GGAATTCAAGTGGCTCCTGAC - 3′ (21mer)
for exon 8
8F :
5′ - GGAATTCCTATCCTGAGTAGTGGT - 3′(24mer)
8R :
5′ - GGAATTCGCTTAGTGCTCCCTGG - 3′ (23mer)

The reaction mixture contained 2.5 mM MgC12, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% of Triton X-100, 0.1 mM each dNTP, 100 pmole of forward and reverse primer, 100 ng of genomic DNA and 1 U of Taq polymerase. PCR reaction was done as 50 μl volume in a 0.2 ml tube. PCR temperature condition was composed of 3 cycles. Cycle 1 was done once with 30 sec at 94℃; cycle 2 was done 35 times with 5 sec at 94℃, 10 sec at 60℃ and 60 sec at 72℃; cycle 3 was done once 120 sec at 72℃. The thermal cycler was Perkin Elmer 2400, USA.

5. Subcloning and sequencing

PCR products were purified with phenol-chloroform (1:1) and DNAs were precipitated with EtOH and 3 M sodium acetate (pH 5.2). DNA pellets were centrifuged at 12,000 rpm (4 ℃). DNAs were digested with EcoRI, electrophoresed in an agarose gel and purified with GeneClean kit II (Bio 101, USA). Purified DNA were ligated into EcoRI-cut pBluescript SKII vector. Ligated DNAs were transformed into *E. coli* by electroporation.

For sequencing, DNAs of pBS containing p53 exons were isolated from transformed *E. coli* DH5α. The 3 - 5 μg of double stranded DNAs were denatured and sequencing reaction was performed with ^{35}S-dATP with T7 and T3 primers as the provider specified. The sequencing reaction mixture was resolved in 6%

polyacrylamide sequencing gel, the gel was dried and exposed onto x-ray film for 50 - 72 hrs.

III. RESULTS AND DISCUSSION

1. Apoptosis by adriamycin in HeLa

In order to monitor HeLa cell apoptosis biochemically, DNA fragmentation assay was performed. A wide range of cell numbers were tested to generate enough DNAs to visualize typical apoptotic DNA fragment ladders. In our experimental condition, 10^7 cells generated enough DNA ladders (data not shown). This number was rather high for DNA fragmentation assay compared to other studies where 5 x 10^5 - 2 x 10^6 cells were used (Huang *et al.*, 1995; Brown *et al.*, 1993). It is not clear whether this represents participation of lower fraction of cells in DNA degradation or simple experimental inefficiency.

Optimum adriamycin treatment condition was screened. Adriamycin concentration range was 0.1 - 100 μM and duration of drug treatment was 0 - 24 hr (Fig. 1). Genomic DNAs started to degrade at concentration more than 1 μM of adriamycin. Thus 1 μM was chosen as concentration condition and optimum time point was selected. DNA ladders ranging 300 - 700 bp were observed starting 3 hr treatment at 1 μM concentration. However the band intensity got stronger up until 12 hr treatment and 24 hour treatment generated overall degradation of genomic DNA. Therefore optimum condition for apoptotic DNA fragmentation assay was 12 hr treatment at 1 μM adriamycin in HeLa cells. This concentration was comparable with that of Skladanoski and Konopa (1993) where 0.98 μM of adriamycin induced apoptosis in HeLa S3 cells.

In order to see any difference in apoptosis induction kinetics between attached cell line and suspension cultured cell line, chronic myelogenous leukemia cell line, K-562 was treated with adriamycin to induce apoptosis. This cell line gave similar apoptotic kinetics to that of HeLa i.e. 12 hr duration at 1 μM adriamycin induced apoptosis (data not shown). Thus our data did not differentiate the apoptosis induction kinetics between attached and unattached cell lines.

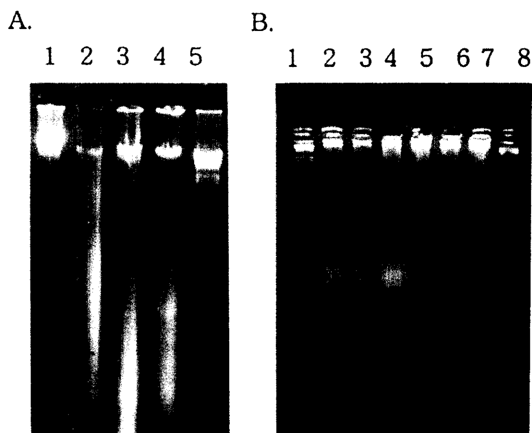

Fig. 1. DNA fragmentation in HeLa by adriamycin. Optimum condition of HeLa cell apoptosis was experimented for various concentrations (A) and times (B). DNA ladders ranging 300-700 bp were observed starting 3hr treatment. Panel A : HeLa genomic DNA with no drug treatment (lane 1); HeLa genomic DNAs after 24 hour adriamycin treatment at 1 μM (lane 2), 10 μM (lane 3), and 100 μM (lane 4) respectively; λ/HindIII DNA marker (lane 5). Panel B : lane 1, λ/HindIII DNA marker; HeLa genomic DNA with no drug treatment (lane 2); HeLa genomic DNAs treated with 1 μM adriamycin treated for 1 hr (lane 3), 3 hr (lane 4), 6 hr (lane 5), 12 hr (lane 6) and 24 hr (lane 7) respectively; lane 8, 123 DNA ladder.

The intravenous dose of adriamycin administration is 0.5 to 1 mg/kg. And the serum concentration could be reached 0.39 - 0.78 μg/ml at t1/2 which corresponds to 0.6 - 1.2 uM for 65 kg man (Goodman and Gilman, 1980). 1 μM is the concentration capable of apoptosis for

HeLa even for 3 hour exposure in this study. Thus 1 mg/kg adriamycin administration could allow the patient's serum drug concentration beyond apoptosis level. However tumor-infiltrating drug concentration colud be lower than the serum drug level. Careful pharmacokinetic study of adriamycin, especially the concentration inside the tumor, is necessary in order to accept apoptosis as a major mechanism of tumor killing.

2. Lack of nucleotide change in mutation hot spots of p53 gene

Mutagens generate specific kinds of base substitutions at certain preferred sites of p53 gene (Thorlacius *et al.*, 1993 ; Spruck III *et al.*, 1993 ; Oda *et al.*, 1992 ; Hollstein *et al.*, 1991). In order to monitor p53 gene status, 3 exons of p53 where mutation hot spots reside were amplified by PCR (Fig. 2). DNA bands of different size were not observed in all three exons of the sample of 12 hr treatment with 1 μM adriamycin. This does not necessarily mean the absence of deletion or addition of nucleotides in that region. The agarose gel system is not sensitive enough to resolve minute size changes.

Therefore for fine dissection of those regions, nucleotide sequence analysis was performed. In order to define the original p53 status before apoptosis, exons of p53 gene from adriamycin-free HeLa cells were also amplified by PCR and sequenced. The nucleotide sequence of adriamycin-free HeLa was the same as the published sequence (Futreal *et al.*, 1991) in all three exons. Three p53 exon sequences from the HeLa cell DNAs of 12 hr treatment with 1 μM adriamycin did not show any changes compared to the p53 sequences from the adriamycin-free HeLa. In order to scan the p53 gene status among HeLa cell population during apoptosis, seven to nine clones for each exon were analyzed. The results are shown in Fig. 3 and summarized in Table 1.

The lack of p53 mutation in hot spots does not guarantee absolute lack of mutation in the entire p53 gene. However this study strongly implies that adriamycin induces apoptosis before reaching a concentration to damage p53 gene mutation hot spots. This results again stands for the safety of adriamycin for its chemotherapeutic action.

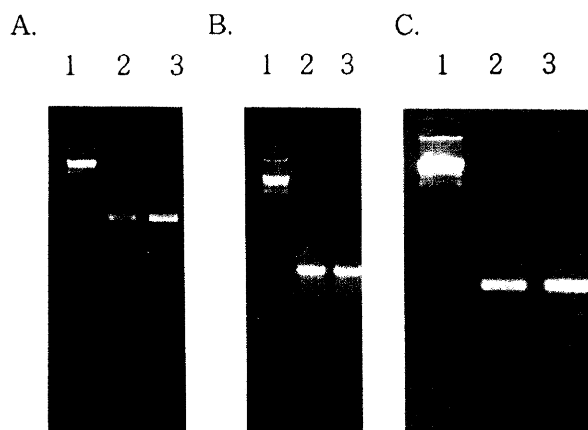

Fig. 2. PCR amplification of exons p53 gene where mutation hot spots reside. PCR products were electrophoresed on a 2% agarose gel. Lane 1 in each panel represents λ/HindIII DNA marker; lane 2 in each panel represents PCR product from HeLa genomic DNA without adriamycin treatment and lane 3 in each panel represents PCR product from HeLa genomic DNA with 12 treatment of 1 μM adriamycin. Panel A, B and C shows PCR products of exon 5 (203 bp), exon 7 (142 bp) and exon 8 (161 bp).

Not many studies deals the selectivity of apoptosis induction by adriamycin in tumor cells and their normal counterparts. As apoptosis becomes a major mode of killing by anticancer drugs, the selectivity issue of apoptosis induction could be an important parameter to evaluate the drug's safety.

Table Ⅰ. Number of sequencing samples of p53 exons in Hela at apoptosis by adriamycin

Exon # of p53	Exon 5	Exon 7	exon 8
No. of clones sequenced	7	9	9

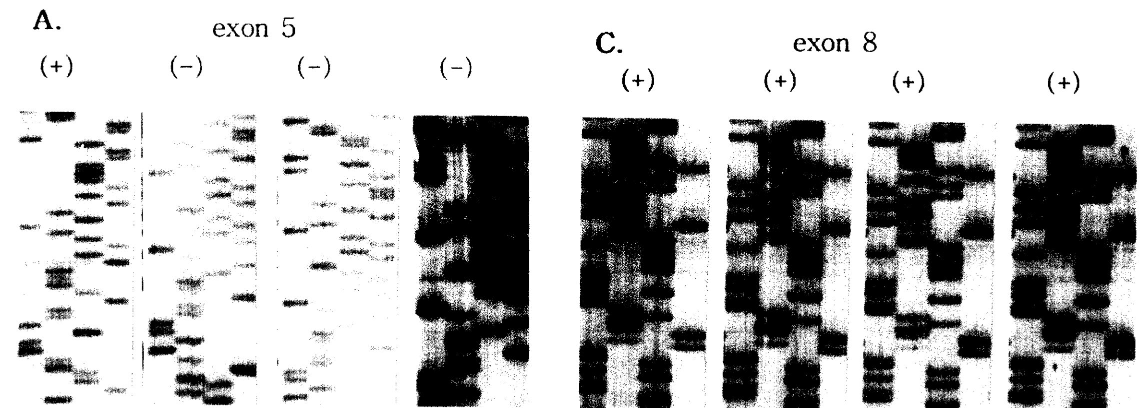

Fig. 3. Mutational Screening of p53 exons in HeLa at apoptosis by adriamycin. p53 exon fragments were amplified by PCR and subcloned to pBS vector and sequences were determined by double strand sequencing method. Panel A, B and C represent examples of sequencing gels from exon 5, exon7 and exon 8. "+" denotes coding strand image and "−" denotes noncoding strand image.

Ⅳ. ACKNOWLEDGMENT

This work was supported by the grant to Eun-Hee Kim for 1996 from Pai-Chai University

Ⅴ. REFERENCES CITED

1. Barry, M.A., Behnke, C.A., and Eastman, A. 1990. Activation of programmed cell death (Apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochemical Pharmacology*, 40: 2353-2362.
2. Brown, D.G., Sun, X.M., and Cohen, G.M. 1993. Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. *J. Biol. Chem.*, 268: 3037-3039.
3. Calabres, P., and Parks, Jr., R.E. 1980. Antiproliferative agents and drugs used for immunosuppression, In; The pharmacological basis of therapeutics (Gilman, A.G., Goodman, L.S. and Gilman, A. eds.) Macmillan publishing Co. USA, 1293.
4. Clarke, A.R., Purdie, C.A., Harrison, D.H., Morris, R.G., Bird, C.C., Hooper, M.L., and Wyllie, A.H. 1993. Thymocyte apoptosis induced by p53-dependent and independent path

-ways. *Nature,* 362: 849-852.

5. Cullinane, C., Rosmalen, A.V., and Phillips, D.R. 1994. Does adriamycin induce interstrand cross-links in DNA ?. *Biochemistry,* 33: 4632 - 4638.
6. Enoch, T., and Norbury, C. 1995. Cellular responses to DNA damage:cell-cyle check-points, apoptosis and the roles of p53 and ATM. *TIBS.,* 20: 426-430.
7. Futreal, P.A., Barrett, J.C., and Wiseman, R. W. 1991. An *Alu* polymorphism intragenic to the TP53 gene. *Nucleic Acids Res.,* 19: 6977.
8. Gaftenhaus, R.B., Wang, P., and Hoffmann, P.1996. Induction of the WAF1/CIP1 protein and apoptosis in human T-cell leukemia virus type I-transformed lymphocytes after treatment with adriamycin by using a p53-independent pathway. *Proc. Natl. Acad. Sci. USA.,* 93: 265-268.
9. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. 1991. p53 mutation in human cancers. *Science,* 253: 252-256.
10. Huang, P., Rovertson, L.E., Wright, S., and Plunkett, W. 1995. High molecular weight DNA fragmentation: A critical event in nucleoside analogue-induced apoptosis in leukemia cells. *Clinicla Cancer Res.,* 1:1005-1013.
11. Kaufmann, S.H., Desnoyer, S., Ottaviano, Y., Davidson, N.E., and Poirier, G.G. 1993. Specific proteolytic cleavage of poly (ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. *Cancer Res.,* 57: 3976-3985.
12. Lowe, S.W., Bodis, S., McClatchey, A., Remington, L., Earl Ruley, H., Fisher, D.E., Housman, D.E., and Tyler Jacks. 1994. p53 status and the efficacy of cncer therapy *in vivo. Science,* 266: 807-810.
13. Lowe, S.W., Earl Ruley, H., Tyler Jacks., and Housman, D.E. 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell,* 74: 957-967.
14. Miyashita, T., and Reed, J.C. 1992. bcl-2 Genetransfer increases relative resistance of S49.1 and WEHI 7.2 lymphoid cell to cell death and fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res.,* 52: 5407-5411.
15. Oda, T., Tsuda, H., Scarpa, A., Sakamoto, M., and Hirohashi, S. 1992. p53 gene mutation spectrum in hepatocellular carcinoma. *Cancer Res.,* 52: 6358-6364.
16. Powis, G., Hodnett, E.M., Santone, K.S., See, K.L., and Melder, D.C. 1987. Role of metabolism and oxidation-reduction cycling in the cytotoxicity of antitumor quinoneimines and quinonediimines. *Cancer Res.,* 47: 2363-70.
17. Skladanowski, A., and Konopa, J. 1993. Adriamycin and daunomycin induce programmed cell death (apoptosis) in tumour cells. *Biochemical Pharmacology,* 46: 375-382.
18. Spruck III, C., Rideout III, W.M., Olumi, A. F., Ohneseit, P.F., Yang, A.S., Tsai, Y.C., Nichols, P.W., Horn, T., Hermann, G.G., Steven, K., Ross, R.K., Yu, M.C., and Jones, P.A. 1993. Distinct pattern of p53 mutations in bladder cancer:relationship to tobacco usage. *Cancer Res.,* 53: 1162-1166.
19. Thorlacius, S., Borresen, A.L., and Eyfjord, J. E. 1993. Somatic p53 mutations in human breast carcinomas in an icelandic population: A prognostic factor. *Cancer Res.,* 53: 1637-1641.
20. Vichi, P., and Tritton, T.R. 1992. Adriamycin: Protection from cell death by removal of extracellular drug. *Cancer Res.,* 52: 4135-4138.