Hacettepe University Journal of the Faculty of Pharmacy
Volume 32 / Number 2 / July 2012 / pp. 119-132

Gene Expression Levels of Apoptotic Proteins and Multidrug Resistance Genes in HEPG2 Cells

Received : 01.01.2013
Revised : 24.05.2013
Accepted : 07.06.2013

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Introduction

In 1979, a new protein which is a Simian virus 40 (SV40) oncoprotein and makes complex with big T antigen is found by researchers[1-4]. This protein is called p53 because of its 53 kDa weight. This tumor inhibitor gene is localised on the short arm of the 17th chromosome[5]. This gene's product, p53 (TP53) protein is a tetrameric nuclear protein protein with 53 kDa weight consisting of 393 amino acids[6-12]. p53 tumor suppressor gene is the most important gene which its role in cancer is well-known and is the most mutated or deleted gene in human cancer cells. p53 gene makes a choice between death, life and apoptosis and prevents the cell from cancer lifelong. As a result of the loss of p53's tumor suppressor activity increases in frequency, size and metastasis of the tumor can be observed[13]. The term apoptosis was used for the first time by pathologists Kerr, Wyllie and Currie in 1972[14,15]. In 1983, the first biochemical evidence of cell death was obtained with gel electrophoresis when it was seen that endonucleases caused 200 bp of DNA fragments and following that studies that concern apoptosis have speeded up[16].

Apoptosis is the programmed cell death which has functions in elimination of unwanted tissues and remodelling of the tissue at the embri-

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ogenesis phase, in provision of development and homeostasis in later years, in aging process, in control of damaged or transformed tissues and when healthy tissues come to the end of their life[15,17,18]. Therefore apoptosis acts as a guardian against cancer by eliminating transformed or potentially harmful cells[19,20]. Over or underexpression of apoptotic proteins can cause ischemia, neurodegenerative diseases such as Alzheimer and Parkinson, chronic diseases such as Acquired Immune Deficiency Syndrome (AIDS), immunologic diseases, viral enfections, tumor growth or regression[21,22]. The apoptotic process is run by caspases also known as cysteine proteases, which cut their substrates from aspartic acid residues and have a role in both extrinsic and intrinsic pathways[23].

A multi-phase plan occurs when cell dies. With the detachment of actin and laminin proteins that forma the cell skeleton, the cell starts to shrink and to shrivel. In the cell nucleus chromatin degradates and the nucleus condenses. Most of the cells take the shape of a horseshoe. Cells get smaller and are packed so that macrophages can swallow them. In the last phases of apoptosis little vesicules can be observed. At the same time, the chromosomal DNA degradates into 180 to 200 bp fragments. This is one of the most significant property of apoptosis[24,25]. Apoptosis (programmed cell death) and necrosis (passive cell death) are two different processes morphologically and biochemically[26,27]. Cell death with apoptosis depends on energy[28], RNA and protein synthesis[29] and it occurs without any cellular damage or inflammation[14,21]. On the other hand, necrosis doesn't depend on energy[14,26] and inflammation is seen[14,30]. The stimulus that triggers apoptosis can be pathological or physiological but necrosis it is always pathological[25,31].

Multiple drug resistance gene-1 (MDR-1) or ATP-binding cassette gene (ABCB1) consists of a central promotor region and 29 exons and is approximately 210 kb long and is located on the long arm of 7th chromosome at the 7q21 choromosomal region[32]. In human genome, there are 2 MDR-1 genes (MDR-1and MDR-2)[33]. MDR-1's relationship withdrug resistance is only identified in human cancer cells[34]. MDR's classical form is overexpression of P-glycoprotein (P-gp) which is a product of MDR-1 gene[35,36]. It is suggested in various studies that P-gp is an energy-dependent pump, it throws out small molecules and anti-cancer drugs out of the cell and it has sequence similarities with hemolysin carrier proteins in bacteria[37].

B cell lymphoma gene-2 (Bcl-2) is located in determinative part of translocation in 14th and 18th choromosomes. Bcl-2 is defined in human B cell follicular lymphoma for the first time[38]. BCL-2 family members control caspase cascade, mitochondria membrane permeability and release of cytochrome c. These are the most important mediators of mitochondria-associated cell death pathway and apoptosis caused by stress[39,40]. In addition, it controls cell survival, the relationship between host and pathogen, animal development and it makes a choice in between life and death[38]. Overexpression of Bcl-2 gene is observed in most of the human cancers[1]. Overexpression of Bcl-2 helps to prevent the toxic effect of anticancer drugs and prevents the cell from undergoing apoptosis[1].

Doxorubicin (DOX), or Adriamycin as the trade name, is a strong anthracycline group of antibiotics used in chemotherapy and in the treatment of solid tumors. It has an anti-tumoral efficiency with a wide spectrum[41-43]. DOX is a drug with anti-neoplastic (cytotoxic) effect which interacts with DNA and resembles daunomycin[44]. DOX is one of the most preferred chemotherapeutic agents used for the treatment of various cancers and especially mammary cancer[45]. DOX causes DNA fractures in the double helix by intercalation, thereby it reveals it's cytotoxic effect by inhibiting macromolecule synthesis[46]. DOX was obtained from *Streptomyces peucetius* in 1960's[47] and it has been used for cancer treatment in the same year[48].

In this study, liver cancer cell line HepG2 and one of the most effective anti-cancer drugs, Doxorubicin (DOX) which is known to stimulate apoptosis were used. HepG2 was exposed to increasing doses of the drug and incubated for 48 hours. We aimed to determine DOX's effect on anti-apoptotic Bcl-2, apoptotic p53 and multiple drug resistance gene MDR-1 and investigate the interaction between these three genes in terms of gene expression levels. HepG2 cancer cell lines are the cell models generally used in anti-cancer drug development studies.

Material and Methods

Cell Lines and Cell Cultures

HEPG2 cells were obtained commercially with DSMZ certificate. The cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM),

supplemented with 10% fetal bovine serum (FBS), 1% Penicilin/Streptomycin and 1% L-Glutamine (L-Glu). Cells were incubated at 37°C with 5% of CO_2 supply.

RNA Purification

HEPG2 cells were plated in 6 cm petri dishes and treated with increasing doses of (50, 200, 400 and 800 nM) DOX. The control group was left untreated. The cells were incubated for 48 hours with the drug. After the incubation period the RNA was purified using a commercial RNA purfication kit (Invitrogen PureLink RNA Mini Kit), according to the manufacturer's protocol. Following the purification, the RNA amount and purity were detected spectrophotometrically (using UV-1800 SHIMADZU spectrophotometer).

cDNA Preparation

1 μg of purified RNA, 1 μl dNTP, 1 μl OligodT were prepared in a total volume of 13 μl and the samples were incubated at 65°C for 5 minutes. Following the incubation, 4 μl of 5X First strand buffer (250 mM Tris-HCl pH:8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 μl DTT, 1 μl RNase inhibitor and 1 μl of Reverse Transcriptase were added. The samples were incubated at 25°C for 5 minutes, 55°C for 60 minutes and 70°C for 15 minutes. All the steps were performed according to the manufacturer's guidelines (Invitrogen SuperScript III CellsDirect cDNA Synthesis System). The cDNAs were kept at -20°C.

Real Time PCR

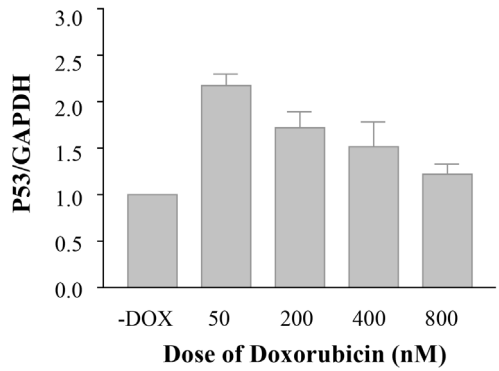
To determine the gene expression levels of p53, Bcl-2 and MDR-1 in HEPG2 cells Real Time PCR was performed using Applied Biosystems 7500 Real Time PCR device. Expression levels obtained for p53, Bcl-2 and MDR-1 genes, were normalized to GADPH.

The primers used were as follows; p53 (Forward CAC GAG CGC TGC TCA GAT AGC and reverse ACA GGC ACA AAC ACG CAC AAA), bcl-2 (forward TGT TTT GAG AGC GTC AAC CG and reverse TCA GGT AGA GGC CGC ATG CTG), mdr-1 (forward AAC TTC AAC TAT CCC ACC CGA CGG and reverse GTA CTG CAG TCA AAC AGA TGG TT) and GAPDH (forward GTC GTA TTG GGC GCC TGG TCA and reverse GCC AGC ATC GCC CCA

CTT GAT). The primers were used at a final concentration of 400 nM. Power SYBR Green PCR Master Mix (Applied Biosystems) was used according to the protocol. The PCR conditions were 32 cycles of 95°C for 30 seconds, 57°C for 45 seconds and 72°C for 45 seconds.

Results

It was observed that in HepG2 cells the gene levels of p53 gene was doubled after 50 nM of DOX administration and gradually supressed upon increasing doses. At 800 nM DOX the gene expression level was close to the control sample (1,225 times compared the control group). The values obtained were analyzed using the statistical analysis software GraphPad and shown at Figure 1.



	Mean	Standard Deviation
0 nM	1	-
50 nM	2,176	0,121
200 nM	1,718	0,245
400 nM	1,524	0,372
800 nM	1,225	0,159

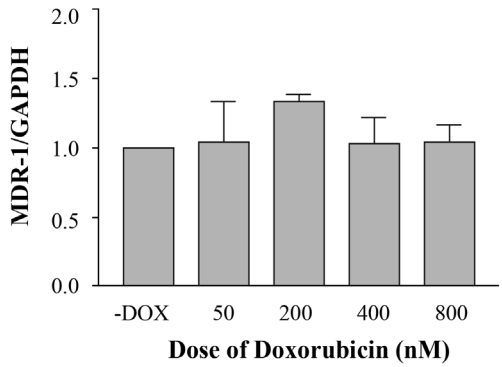
Figure 1
Gene expression levels of p53 gene in HepG2 cells (n=3)

The gene expression values for mdr-1 after DOX incubation was shown at Figure 2. It can be observed that the gene expression levels for mdr-1 are independent from the dose administrated to the cells.

In HepG2 cells, the expression levels of bcl-2 gene have dramatically increased in accordance with the dose administrated (6,374 times compared to the control group at 200 nM) but then decreased visibly as the dose increased (5,641 and 3,9120 times compared the control group at 400 and 800 nM of doxorubicin, respectively).

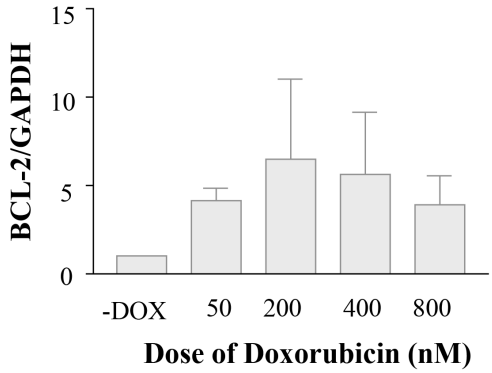
Conclusions

Chemotherapy is one of the most efficient methods used to treat cancer. In this study, the drug DOX which is a popular anticancer agent in the treatment of various cancer types was used to understand the interaction between apoptotic pathways and multidrug resistance in HEPG2



	Mean	Standard Deviation
0 nM	1	-
50 nM	1,042	0,293
200 nM	1,332	0,070
400 nM	1,029	0,267
800 nM	1,036	0,179

Figure 2
Gene expression levels of mdr-1 gene in HepG2 cells (n=3).



	Mean	Standard Deviation
0 nM	1	-
50 nM	4,182	0,700
200 nM	6,512	6,374
400 nM	5,641	4,961
800 nM	3,910	2,296

Figure 3
Gene expression levels of bcl-2 gene in HepG2 cells (n=3).

cells. Clinically, various cancer cells such as liver cancers cells are sensitive when they are first exposed to chemotherapeutic drugs but they develop drug resistance afterwards. Resistance of tumor cells to cytotoxic drugs is defined as multiple drug resistance (MDR). MDR-1 gene is responsible for the development of MDR in humans. For this reason, the studies that concern MDR are highly important.

Apotosis is stimulated by p53 protein or gene activated with DNA damage or other factors[49]. The ratio of the genes that prevent apoptosis to apoptotic genes (BCL-2/BAX) and p53 levels determine the destiny of the cell[50]. Other than these, the apoptotic process is run by caspases. Therefore, mitochondria play an important role in all apoptotic processes[23].

p53 is mutated in approximately 50% of cancer types and 90% of these mutations occur especially in the central DNA binding unit[13,51]. For these reasons, p53 is in the center of cancer research studies. p53 has some critical and important functions in cellular processes such as gene transcription control, DNA repair[52], cell cycle control, genomic stability, apoptosis and tumor suppression[13,52].

Bcl-2 is located in the translocation determinative part of the 14th and 18th choromosomes. It was first identified in human B cell follicular lymphoma[43]. The Bcl-2 family members are known to modulate caspase cascade, mitochondria membrane permeability, mitochondrial death pathway which controls cytochrome c release and apoptosis which is caused by stress[43-45]. In this study, Bcl-2 gene was studied from the group of Bcl-2 family's anti-apoptotic proteins. It is well known that MDR development after DOX treatment occurs with the increase of E-cadherin and P-gp expression levels with the increase of cell adhesion[53,54]. It is reported that MDR-1 gene is stimulated in mammary cancer cells with the accumulation of mutant p53[55]. According to a study, pro-caspase-3 activation is significantly increased after treatment of DOX for 48 hours[56].

In this study, RT-PCR method was carried out to investigate the changes of p53, Bcl-2 and MDR-1 gene expression levels in HEPG2 cells and the data was evaluated statistically. It was observed that, p53 gene expression levels were doubled after administration of 50 nM of drug in HEPG2 cells. On the other hand, the expression levels were decreased with increasing doses of the drug but the expression levels were still higher compared to the control group. It was observed that, p53 gene expression levels were increased with administration of DOX, therefore it was ended up with the accumulation of p53 protein[56]. Furthermore, it was also seen that the increase in p53 gene expression with increasing doses of DOX was less, compared to gene expression levels in cells treated with low doses of the drug. Gene expression experiments showed that the highest dose of DOX that was administrated in these experiments (800 nM) gave almost the same data with the expression levels of the control (to 1,225 times). With the data obtained from this study, it was understood that mdr-1 gene expression levels are independent from the doses of DOX administrated to HEPG2 cells. HEPG2 cells' viability was decreased with increasing doses of DOX.

It was observed that Bcl-2 gene expression genes were significantly increased with increasing doses of DOX at first, but decreased when the dose of administration kept increasing. It is known that normally Bcl-2 gene expression levels are decreased as a result of increase of p53 gene expression levels. Therefore, it is thought that p53 leads increase in Bcl-2 quantity in cells and stimulates apoptosis with increasing doses of DOX. Graphics that were obtained from the results show that expressions of

two genes are consistent with each other and with the literature. It was observed that p53 levels were increased with 50 nM of DOX, but at higher doses of the drug the expression levels were decreased. It was also observed that Bcl-2 gene expression levels were significantly increased with administration of 50 nM of DOX and this increase was also seen with 200 nM of drug administration compared to the control group. With these results, it is estimated that Bcl-2 increases its gene expression levels and suppresses p53's increase of expression levels.

When the results of three gene expression levels in HepG2 cancer cell lines were evaluated together, it is thought that Bcl-2 and p53 genes mediate each other's gene expression levels in HepG2 cells and as a result, they develop a different apoptotic response. But at the gene level the multidrug resistance is unaffected from the increasing doses of doxorubicin administrated to the cells. These results suggest that in HepG2 cells, the apoptotic response and the multidrug resistance are controlled by independent mechanisms, rather than together. Still, these data should be evaluated further, using various cancer cell lines and by comparing their apoptotic and multidrug resistance profiles. Such a study may provide us a pattern to predict the success of novel chemotherapeutic agents in different cancer types and help us develop suitable candidate molecules.

Acknowledgement

This study was granted by the Scientific Research Unit of Hacettepe University, H.Ü.B.A.B (Project Number: 0801301002).

Summary

In this study, liver cancer cell line (HepG2) were used with the aim of examining the apoptotic-antiapoptotic mechanisms and multi drug resistance pathways which are defective in cancer cells. The aim of this study: is to trigger apoptosis by treatment HepG2 cancer cell line with one of the most effective anticancer drug Doxorubicin and to investigate the expression levels of p53, Bcl-2 and MDR-1 genes which are related with each other and play an important role in apoptosis. With this purpose,

the cells were grown by treating with increasing doses of Doxorubicin. Their RNA's were purified, converted into cDNAs and used in Real Time PCR device in order to determine the expression levels of these genes. It is observed in HepG2 cells, compared to the control groups after 50 nM, 200 nM, 400 nM ve 800 nM drug treatment, p53 gene expression level is respectively 2,176, 1,718, 1,524, 1,225 fold increased, Bcl-2 gene expression level is respectively 4,182, 6,512, 5,641, 3,910 fold increased and MDR-1 gene expression level is not dependent to the treated drug dose.

Keywords: Gene expression, p53, bcl-2, mdr-1, HepG2

Özet

HEPG2 Hücrelerinde Apoptotik Proteinlerin ve Çoklu İlaç Direnci Geninin Ekspresyon Düzeyleri

Bu çalışmada, kanser hücrelerinde işleyişi hasarlı olan apoptotik, anti-apoptotik mekanizmaların ve çoklu ilaç direnci yolakların incelenmesi amacıyla, karaciğer kanseri hücre hattı (HepG2) kullanılmıştır. Bu çalışmanın amacı: HepG2 hücre hattını en etkili antikanser ilaçlardan biri olan Doksorubisin ile muamele ederek, apoptozun uyarılması ve apoptozda önemli rol oynayan birbiriyle ilişkili p53, Bcl-2 ve MDR-1 genlerinin ekspresyon düzeylerinin incelenmesidir. Bu amaçla hücreler ilaç ile muamele edilerek büyütülmüş, sonrasında RNA'ları saflaştırılmış, cDNA'ya dönüştürülmüş ve elde edilen cDNA'lar üç genin ekspresyon düzeyini belirlemek amacıyla RT-PCR cihazında/yönteminde kullanılmıştır. HepG2 hücrelerinde 50 nM, 200 nM, 400 nM ve 800 nM ilaç uygulanmasından sonra kontrol gruplarına kıyasla deney gruplarında, p53 geninin ekspresyon düzeylerinin sırasıyla 2,176, 1,718, 1,524, 1,225 kat arttığı, Bcl-2 gen ekspresyon düzeylerinin sırasıyla 4,182, 6,512, 5,641, 3,910 kat arttığı ve MDR-1 gen ekspresyon düzeyinin ise uygulanan ilaç dozundan bağımsız olarak değişmediği gözlenmiştir

Anahtar kelimeler: Gen ekspresyonu**,** p53, bcl-2, mdr-1, HepG2

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