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İNSAN MULTİPLE MYELOMA HÜCRE DİZİSİ ARH77'DE VİNKRİSTİNİN SURVİVİN GEN İFADESİ ÜZERİNE ETKİSİ

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ÖZ:

Amaç: Apoptozis İnhibitör Gen ailesinin (IAP) bir üyesi olan survivin, hücre bölünmesi, apoptozis ve strese hücresel yanıt gibi birçok olayda rol oynayan mitotik bir proteindir. Multiple myelomada (MM) temel kemoterapi ajanlarından biri olan vinkristin, hücrelerde mikrotubül olusumunu engelleyerek apoptozisi uyarmaktadır. Bu çalısmada, MM hücre dizisi ARH77 hücrelerinde vinkristinin doz ve süre bağımlı olarak uygulanması ile survivin gen ifadesi üzerinde olusturabileceği değisiklikleri incelemeyi amaçladık.

Gereç ve Yöntem: İnsan MM hücre dizisi ARH77 standart kültür kosulları altında değisen konsantrasyonlarda (10^{-7}- 10^{-10} M) ve değisen sürelerde (24, 48, 72 ve 96 saat) vinkristine maruz bırakıldı. Survivin gen ifadesi kantitatif RT-PCR ile belirlendi. İstatistiksel olarak gruplar arası farklar iki oran z testi ile değerlendirildi.

Bulgular: 24 ve 96 saat uygulama ile survivin gen ifadesinde artıs belirlenmedi. 48 ve 72 saatlik uygulama sonrası görülen gen ifade artısı istatiksel açıdan anlamlı idi. ($p<0,05$)

Sonuç: Vinkristin uygulanan hücrelerde, survivin gen ifadesine etkisi doza ve süreye bağımlıdır. Vinkristinin uyardığı survivin asırı ifadesine bağlı olarak hücrelerde meydana gelecek değisiklikleri moleküler düzeyde inceleyen daha detaylı çalısmalara ihtiyaç vardır.

Anahtar Kelimeler: Survivin, ARH77, Vinkristin.

VINCRISTINE INDUCED SURVIVIN EXPRESSION IN THE HUMAN MULTIPLE MYELOMA CELL LINE ARH77

ABSTRACT:

Purpose: Survivin, an inhibitor of the apoptosis (IAP) gene family, is a mitotic protein with actions on various cellular events such as cell division, apoptosis, and cellular response to stress. Vincristine, a chemotherapeutic agent used in multiple myeloma (MM), induces apoptosis via inhibition of spindle formation. We aimed to detect the expression pattern of survivin in vincristine treated MM cell line ARH77.

Materials and Methods: Vincristine was added to human multiple myeloma cell line ARH77 cultures for 24, 48, 72, and 96 h and 10^{-7} to 10^{-10} M concentrations. Survivin expression was determined by quantitative RT-PCR. Statistical comparisons of ratios were made with two proportions z test.

Results: We did not observe an increase in survivin expression after 24 or 96 h of treatment. Survivin expression significantly increased after 48 and 72 h ($p<0,05$)

Conclusion: Expression of survivin in vincristine treated cells is dependent on time and concentration. Detailed studies are needed to evaluate changes in vincristine induced survivin overexpression in cells at the molecular level.

Key words: Survivin, ARH77, Vincristine.

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INTRODUCTION:

Multiple myeloma (MM) is a malign B cell disease characterized by an accumulation of malign plasma cells in bone marrow. Cell cycle regulating proteins such as cyclin D and cyclin dependent kinases and chromosome abnormalities such as aneuploides and translocations play role in disease pathogenesis. In the clinically stable phase of the disease apoptosis defects cause accumulation of myeloma cells in bone marrow.[1,2] In addition to melphalan and prednisone, vincristine, adriamycin, doxorubicin, and dexamethasone are among the chemotherapy regimens for MM. All patients with MM eventually require chemotherapy; however, treatment is far from successful, as patients frequently develop drug resistance.[3]

Vincristine is a naturally occurring antimicrotubule agent and induces the destabilization of polymerized tubulin by blocking the region involved in tubulin dimer attachment, therefore preventing polymerization of microtubules. It has been generally thought that it blocks cell cycle progression in the G2-M phase.[4]

Recently, a new family of proteins termed inhibitor of apoptosis proteins (IAPs) has been discovered, which are involved in inhibition of caspases 3, 7, and 9.[5,6] Survivin is the smallest member of the IAP family. It has been mapped to the chromosomal region 17q25, and encodes a 1.9-kb transcript, which contains[4] exons resulting in a 142-amino acid (16.5 kDa) protein. Alternative splicing of the human survivin gene can give rise to different mRNA isoforms.[6]

From the biological standpoint, survivin is a multifunctional protein that has been demonstrated to inhibit apoptosis, regulate cell division, and promote angiogenesis.[6] It is involved in the preservation of mitotic spindle integrity.[5] Multiple studies have shown that survivin inhibits apoptosis in response to a wide variety of apoptotic stimuli. Recent evidence suggests that survivin may inhibit apoptosis by binding to and inactivating second mitochondria-derived activator of caspase (SMAC), a mitochondrial protein that potentiates apoptosis by complexing with specific IAPs, thus preventing their interaction with caspases.[6] In many tumor cells, including hematopoietic neoplasms, survivin is one of the highly expressed genes.[7]

Survivin gene is preferentially expressed in the G2-M phase of the cell cycle. Treatment of cells with taxol, a microtubule depolymerization inhibiting agent, may induce M phase arrest and upregulate transcriptional activity of the survivin

promoter thereby increasing survivin expression.[8] In a recent study, Kawamura et al. demonstrated that taxol treatment influenced the transcriptional activity of the survivin promoter whereas vincristine did not.[9] It has been suggested that cell cycle dependency of survivin expression could in part explain its elevated expression in tumors but it was presumed that the transcriptional activation is not directly linked with cell proliferation.[8,9]

Human cell lines sharing characteristics with B-lineage cells obtained from patients with plasma cell dyscrasia may act as useful in vitro models for the study of MM. Furthermore, myeloma cell lines have been used as targets for antitumor chemotherapy, immunotoxins, and oligonucleotide anti-sense strategies and for investigating drug resistance.[3,10,11] In the current study, we aimed to investigate the effect of vincristine on survivin expression in a MM cell line, ARH77, in a time and dose dependent manner.

MATERIALS AND METHODS

Cell cultures: Human MM cell line ARH77 cells were cultured in 24-well culture dishes in RPMI culture medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 2 mM L-Glutamine in an incubator in 5% carbon dioxide and 95% humidity at 37 °C.

Cell viabilities were evaluated by trypan blue dye exclusion test. Every well of the 24-well culture dish contained at least 10^5 cells with 98% viability. Cultures were incubated with changing concentrations of vincristine between 10^{-7} and 10^{-10} M for 24-96 h.[4,12]

Total RNA isolation: Total RNA was isolated using High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Reverse Transcription: Complementary DNA (cDNA) was preperad by first strand cDNA synthesis kit for RT-PCR (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer.

Real time (RT) PCR: A quantitative real time RT-PCR light cycler (Roche Diagnostics, Mannheim Germany) kit was used in the RT-PCR reactions. Survivin gene expression was detected by using previously reported common variant primers and probes.[13] The primer and probe sequences are shown in Table 1. The number of gene copies was calculated by the LightCycler Software Version 1.0 according to the Fit Points Above Threshold method. Real time PCR assays were conducted in duplicate for each sample and the mean value was used for calculation of the relative expression level. The final expression level of survivin mRNA was expressed as ratios to those of G6PDH. The results were normalized to untreated control cells.

Table 1: Primer and probe sequences used in the study

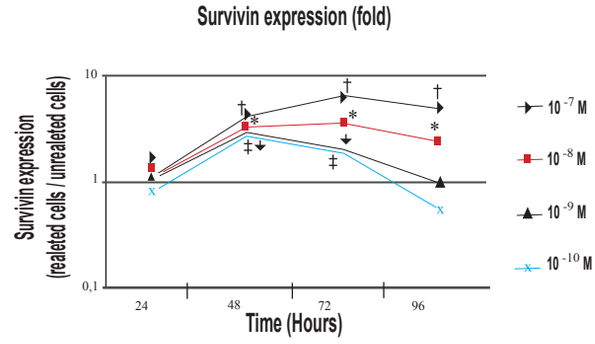
Gene	Primers	Probes
Survivin	Forward 5′ CCACCGCATCTCTACATTCA-3′ Reverse 5′- TATGTTCCTCTATGGGGTCG-3	5′-CAAGTCTGGCTCGTTCTCAGTGGG-3′-FITC LC Red 640-5′-CAGTGGATGAAGCCAGCCTCG-3′
G6PDH	Forward 5′GGACCTGACCTACGGCAACAGATA-3′ Reverse 5′-GCCCTCATACTGGAAACCC-3′	5′-TTTTCACCCCACTGCTGCACC-3′-FITC LC Red 640-5′-GATTGAGCTGGAGAAGCCCAAGC-3′

Statistical analyses: Two proportions z test was used in comparing percentages of cells. $p<0.05$ was regarded as statistically significant. The MINITAB 13.0 statistics package was used in the analyses.

RESULTS

Quantitative RT-PCR results: Survivin mRNA levels are shown in Figure 1. The highest level of survivin expression was observed in 10^{-7} M applied concentrations of vincristine. Survivin upregulation was less induced by decreasing concentrations of vincristine. For all concentrations, 24 h incubation did not induce survivin expression. Survivin expression increased with all doses of vincristine for 48 and 72 h when compared to untreated control cells ($p<0.05$). Finally 96 h incubation with 10^{-7} and 10^{-8} M concentrations of vincristine resulted in a significant increase in survivin expression ($p<0.05$) when compared to untreated controls.

Survivin expression reached the highest levels after incubation with 10^{-7} M concentrations of vincristine for 72 h (up to six-fold compared to the controls). For this concentration, when the incubation time reached 96 h, survivin expression was induced less ($p<0.05$). With 10^{-8} M vincristine, the second highest concentration used in our study, after incubation for 48, 72, and 96 h, survivin expression increased three- to four-fold compared to the controls levels. For all incubation periods, 10^{-7} M vincristine resulted in a significant increased in survivin expression when compared to 10^{-8} M concentration ($p<0.05$).



†,↓,∗,‡ indicates statistically significent increased survivin expression for 10^{-7},10^{-8},10^{-9},10^{-10} M concentrations of vincristine, mapectivelly (p 0.05)

DISCUSSION

Vincristine, which is used in MM chemotherapy combined with other chemotherapeutics such as adriamycin and dexamethasone, inhibits microtubule formation during mitosis.[14] It starts the apoptotic pathway via caspase activation, DNA fragmentation, cytochrome C, SMAC r elease from mitochondria, and nuclear translocation of apoptosis inducing factor (AIF).[10] It causes cell cycle arrest in the G2-M phase.[4] It has been demonstrated that cellular response against the effects of apoptotic inducers (therapeutics) such as vincristine at the molecular level includes increased survivin expression in different cell lines.[9,10,15]

Survivin is a multifunctional protein that has been demonstrated to inhibit apoptosis, regulate cell division and promote angiogenesis.[6] Synthesis and degradation of survivin is modulated in a cell cycle dependent manner. Its transcription increases in G1 and reaches the maximum level at G2/M. This denotes survivin's role in mitosis.[16] Survivin may inhibit apoptosis in several ways such as binding to and inactivating SMACs thus preventing their interaction with caspases.[6] Another way is that, it may also inhibit apoptotic pathways independent of caspases.[10] Thus, the interaction of survivin and caspases is still controversial.

It has been shown that survivin promoter was not modulated by vincristine;[9] on the other hand, vincristine doses between 0.01 - 1.0 μM were reported to be effective in pediatric anaplastic large cell lymphoma cell line and human breast cancer cell line, BCap37.[4,12] Furthermore, in Bcap37 cells 5 X 10^{-8} M vincristine induced apoptosis and increased survivin protein level alone or when combined with γ-radiation.[4]

We incubated our myeloma cell line cultures with similar doses changing between 10^{-7} and 10^{-10} M concentrations. Consistent with Sui et al., we detected the highest survivin expression in 10^{-7} M concentration.[4] The highest level of survivin was detected after 72 h of incubation. The level increased up to six-fold when compared to untreated control cells. 10^{-7} M vincristine increased survivin expression after 96 h but the increase was not as much as after 72 h of incubation. Other concentrations and incubation periods of vincristine caused a slight but significant increase in survivin expression when compared to the untreated control cells. Our study showed that dramatically increased survivin expression was observed with 10^{-7} M and higher concentration of vincristine.

Patients with MM frequently develop drug resistance. Overexpression of survivin is associated with drug resistance and poor prognosis in MM.[3] Our results showed that survivin was overexpressed when cells were treated with vincristine. Development of drug resistance depends on several molecular mechanisms. According to our results, we suggest that survivin expression interacts with apoptotic mechanisms decreasing cellular response by inhibiting apoptosis rather than developing drug resistance.

Some previous studies have investigated the relationship between MM and survivin.[3,13,17] Increased survivin expression has been detected in bone marrow, peripheral blood, or pleural effusion fluid cells of MM patients.[3] In the current preliminary study, we chose vincristine as a component of the chemotherapy protocols in MM. However, without assessing apoptosis related markers such as caspases, we found that survivin expression is dependent on vincristine treatment. We think it is an important finding as it has been reported that in MM patients *(in vivo)* increased survivin expression inhibits apoptosis.[3] Further detailed studies are needed to evaluate changes dependent on vincristine induced survivin overexpression on cells at the molecular level.

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