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Effects of Three Actinic Keratosis Mutations on Apoptosis

[Üç Aktinik Keratoz Mutasyonunun Apoptoz Üzerine Etkisi]

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ABSTRACT

Introduction: Ultraviolet radiation contributes to the etiology of skin cancers by generating mutations in the *p53* tumor suppressor gene. The incidence of skin cancer is doubling each decade; 95% of these are the non-melanoma skin cancers. Actinic keratosis is a precancerous lesion characterized by aberrant proliferation and cell differentiation that progresses to squamous cell carcinoma at a rate of ~0.1% per year. Our aim was to determine whether mutant p53 alleles found in Actinic Keratosis have different phenotypic effects on UVB-induced apoptosis that might explain why only some progress to squamous cell carcinoma.

Material and Method: Three point mutations were generated on an expression vector by site-directed mutagenesis and then transiently transfected into primary mouse fibroblasts. Apoptosis was induced by UVB radiation and detected by flow cytometry.

Results: We found that mutation L201F behaved like the wild type P53 vector. This region retains transcriptional activity in yeast. In contrast, G245S showing no transcriptional activity and is skin tumor hotspot induced exceedingly high spontaneous apoptosis, similar to the maximum level of UV-induced apoptosis in untransfected cells. Little additional apoptosis was induced by ultraviolet. The mutant A84V showed intermediate behavior.

Discussion: The frequency of an AK mutation in non-melanoma skin tumors thus correlated with the extent of reduced transcriptional activity and elevated apoptosis, with the latter occuring either spontaneously or after ultraviolet exposure.

Key Words: p53, Mutation, Apoptosis, UVB, Actinic keratosis

ÖZET

Giriş: Her sene cilt kanseri vakaları artmaktadır ve bu kanserlerin % 95'ni nonmelanoma cilt kanserleri oluşturmaktadır. Güneş yanığı hücreleri olarak da bilinen aktinik keratozlar bir nonmelanoma cilt kanseri çeşitidir. Güneş ışığının önemli kısmını oluşturan ultraviyole, cilt kanserlerinin etiyolojisine büyük ölçüde katkıda bulunmaktadır. Ultraviyole mutasyonlara sebep olan DNA fotoürünlerini oluşturmak suretiyle DNA'da hasarlar yaratmaktadır. P53'ün kanserlerin % 50'sinden fazlasında mutasyona uğradığı ve bu mutasyonların büyük kısmının da yanlış kodlanmış nokta mutasyonlar olduğu bilinmektedir. Bu çalışmada amacımız aktinik keratozlarda bulunan p53'ün farklı mutant allellerinin ultraviyoleye'ye bağımlı apoptoz üzerine etkilerini tespit etmekti.

Gereç ve Yöntemler: Bunun için bir dizi biyoinformatik çalışma sonucu 3 nokta mutasyonu tespit edilmiştir. Bu mutasyonlar ekspresyon vektörü üzerinde bölge yönelimli mutagenezle yaratılmıştır. Mutant ve yabanıl tip vektörler primer fare fibroblastlarına transfekte edilmiş ve 1000 J UVB radyasyona maruz bırakılmıştır. Apoptoz yüzdesi flow cytometry'le ölçülmüştür.

Sonuçlar: Çalışmamızın sonucunda L201F mutasyonunun etkisiz davrandığı G245S mutasyonunun yüksek endojen apoptoza sahip olmasına rağmen ultraviyoleye bağımlı apoptoz yüzdesinin düşük olduğu görülmüştür. A84V'de artmış endojen apoptoza rağmen ultraviyoleye bağımlı apoptoz açısından yabanıl tip gibi davranmıştır. Mutasyonun kazandığı fiziksel ve kimyasal değişiklik, evrimsel olarak korunmuş bölgede yer alması ve/veya DNA'ya bağlanma bölgesine yakınlığı bu mutasyonların apoptoz açısından davranışını etkilemektedir.

Anahtar Kelimeler: p53, mutasyon, apoptoz, UVB, aktinik keratoz

Introduction

Apoptosis, a form of programmed cell death characterized by cell shrinkage, membrane blebbing, nuclear breakdown and DNA fragmentation, is essential for development, maintenance of tissue homeostasis, and elimination of harmful cells [1]. The presence of toxic agents, DNA damage, death receptor signals, ectopic expression of nuclear oncogenes, and the withdrawal of survival signals such as growth factors, cytokines, or hormones are factors that initiate apoptosis [2]. The tumor suppressor P53 is a key regulator that mediates apoptosis.

P53 is a sequence specific transcription factor responsible for maintaining the integrity of the genome [3]. It is a tetrameric protein that has a very short half-life. Therefore in undamaged cells the levels of P53 remains very low [4]. Mdm-2, which is one of the downstream target elements of P53, is responsible for keeping P53 at low levels by ubiquitinating and mediating the degradation of the protein [5]. Depending on the stimuli, P53 contributes to the activation of two major pathways that control tumor growth: the arrest of the cell cycle and the regulation of apoptosis [6]. After cellular exposure to various DNA-damaging agents (such as ionizing radiation, UV radiation, chemotherapeutic agents, hypoxia, or virus infection) P53 accumulates and activates the transcription of several downstream target elements [3, 4].

P53 is known to be mutated in more than 50 % of human cancers [7]. In the latest version of the International Agency for Research on Cancer TP53 Mutation Database (released in November 2007) **23,544 somatic mutations and** 376 germline mutations have been reported where 73.7 % of these are missense mutations. Most of these mutations occur in the DNA binding domain of the protein, especially on the evolutionarily conserved regions. *P53 is inactivated by allelic loss, small deletions and point mutations [8]. Some point mutations may confer characteristics to the protein such as gain of function, loss of function or dominant-negative properties [9].*

Ultraviolet radiation (UV) is the major carcinogenic component of sunlight, a carcinogen to which everyone is exposed. In the light spectrum it lies between X-ray and visible light and is divided into three regions: UVC, 100-280 nm, UVB, 280-320 nm and UVA, 320-400 nm. Although UVC has the shortest wavelength, thus the highest energy, it is completely absorbed by the ozone layer. UVB causes the greatest DNA damage. It induces cyclobutane dimers between two adjacent pyrimidine bases on the same DNA strand [10]. Fortunately, most of these genetic lesions are corrected very shortly after they are created, before they can lead to a mutation. The nucleotide excision repair system is one of the mechanisms which the organism uses to repair the damaged portion of the DNA [11]. The resulting mutations have a distinctive mutation signature: C to T transitions at dipyrimidine sites, with CC to TT substitution in 10 % of the cases [12].

The skin, as the largest organ of the body and exposed to sunlight, is subject to developing cancers as a result of accumulating UV-induced mutations. Skin cancers are classified as melanoma and nonmelanoma. Every year the incidence of skin cancer is increasing and 95% of these are nonmelanoma skin cancers. Unlike the melanomas that originate from melanocytes, the basal cell carcinoma (BCC) develops from hair follicles and squamous cell carcinoma (SCC) originates from the basal layer of the epidermis. They develop primarily on sun-exposed areas of the body, such as the face, ear, neck, lips, and the backs of the hands. BCCs are spread by local invasion and tend to remain diploid. SCCs are less frequent but are more aggressive; they have a greater tendency to metastasize and they become aneuploid. AKs are characterized by aberrant proliferation and cell differentiation. If left untreated, AKs will often regress but develop into an SCC [13]. Therefore AKs are thought to be the first step in the development of skin cancer. It is thus a *precursor* of cancer.

The aim of the present study was to determine whether mutant p53 alleles found in AK have different phenotypic effects on UVB-induced apoptosis that might explain why only some AK progress to SCC. For this purpose, three mutations found in AK lesions were generated by site directed mutagenesis on an expression vector. The wild type and mutated vectors were transiently transfected into mouse primary fibroblast cells.

Materials And Methods

p53-Mutant Expression Vectors. pRcCMVp53WT was digested with *Xba*I and the *p53* cDNA fragment ligated into the XbaI site of vector pEGFP-f (Clontech, Mountainview, CA) upstream of the EGFP promoter. The pEGFP-f-WTp53 vector was sequenced to confirm the p53 cDNA orientation. Three AK mutations were created on the template pEGFP-f-WTp53 using the QuikChange Site Directed Mutagenesis Kit (Stratagene, LaJolla, CA), according to the manufacturer's instructions. These mutations were A84V, L201F and G245S.

Primary Murine Fibroblasts. The primary mouse fibroblasts were preferred over cell lines (such as HeLa, HACaT, or Saos2) because these immortalized cells are already defective in apoptosis and cell cycle arrest. After transfection, the cells were irradiated with UVB, harvested, and assayed for apoptosis. Dermis from newborn mice was separated from epidermis and placed in a Petri dish containing 5 ml collagenase type I (0.35% wt/vol) (Sigma, St. Louis, MO) and minced by scissors into small pieces. The solution was incubated at 37ºC for 45 minutes, then filtered through a cell strainer and centrifuged at 500g for 5 minutes. The cells were resuspended in DMEM high glucose (GIBCO-BRL, Carlsbad, CA) with 10% fetal bovine serum and 1% penicillin/streptomycin. The suspension was then placed in a 150 cm^2 flask and grown at 37ºC, 5% CO_2 until cells reached 60-80% confluency.

Transfection of the Expression Vector into Primary Mouse Fibroblast Cells 16 hours prior to transfection, 350,000 fibroblast cells no older than 5th passage were plated at 60% confluency in antibiotic-free DMEM media in 6 cm petri dishes. 16 hours later the cells reached 60-70 % confluency. The cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a ratio of 1:3 (vol:vol), according to the manufacturer's instructions. The vector was expressed 48 hours post-transfection. Each set of transfections included cells transfected with empty vector and cells transfected with a wild-type P53 vector. The vector EGFP-WTp53 was transfected into fibroblast cells to obtain the samples named "Wild Type p53 (WT p53)". The expression vector containing the corresponding mutation (named "MUT p53") was used to test each mutation. 72 hours post transfection; the media of the cells was replaced with 1 X PBS. Half of the cells were not irradiated (named "NO UV WT" and "NO UV MUT") and their media was replaced with fresh DMEM. The remaining cells were subject to UVB radiation and named "UV WT" and "UV MUT" according to the vector with which they were transfected.

Confirmation of the Point Mutation Generation Using Sequencing. After generating the point mutations by site directed mutagenesis the results were verified by using an automated sequence analyzer. Sequencing Primer-1 (5'-TGAGTCAGGAAACATTTTCA-3' and 5'-CGTCATGTGCTGTGACTGCT-3') and Sequencing Primer-2 (5'-TCTGTGACTTGCACGTACTC-3' and 5'-GGGCAGCTCGTGGTGAGGCT-3') were used. The sequence analysis confirmed all mutations.

UV Irradiation. 20 hours before collecting the samples for the annexin assay, the cells were washed twice with 1X PBS and sufficient PBS to cover the cells was added. The cells were exposed to UVB from 3 broadband FS20T12-UVB lamps filtered through a Kodacel filter (Eastman Kodak, Rochester, NY) to remove wavelengths below 290 nm. The lamp output was 250-420 nm with peak emission at 313 nm, and after filtering contained 72.6% UVB, 27.4% UVA, and 0.01% UVC as measured by an IL1700/790 spectroradiometer with double monochromator (International Light, Inc., Newburyport, MA). The dose rate was 2.2 J/m^2/sec. The cells were covered by a UVB filter so that the final lamp output was >99% UVB. After irradiation, 1X PBS was replaced with regular DMEM containing FBS and penicillin/streptomycin antibiotic. The UVB output of the lamp was measured prior to each session using a UVX meter (UV Products, Upland, CA). The dose response study was carried out with fibroblast cells in order to demonstrate the effect of the UVB dosage on apoptosis. Primary mouse fibroblasts (350000 cells in 6 cm petri dishes) were plated 16 hours prior to radiation. The day of the experiment 4 of the 5 the samples were irradiated with 250, 500, 750 and 1000 J of UVB and one sample was left non-irradiated. The cells were then collected by trypsinization (including the media containing the floating cells) and subjected to Annexin assay.

Apoptosis Measurement by Annexin Assay and Flow Cytometry. The Vybrant 6 apoptosis kit was used to perform the Annexin assay. The media containing floating cells was also collected. All the samples were incubated with Alexa Fluor 350 and propidium iodide. The controls were incubated separately with the proper fluorescent dye. The cells were analyzed using an LSR-II flow cytometer (BD Biosciences, San Jose, CA). Forward and side scatter signals were set using cells without transfection, UV, or fluors. FL1 was set using cells transfected with EGFP only; FL2 using untransfected cells irradiated with 1000 J/m^2 UVB and treated with Alexa Fluor 350; and FL3 using untransfected cells irradiated with 1000 J/m^2 UVB and treated with propidium iodide. Apoptosis values for the empty vector were subtracted from values for the *p53*-containing vectors. The output of the LSR-II Flow Cytometer sample tubes were recorded as ".fcs" extension files. The files were read and evaluated in FlowJo (version 5.1), a Java based software.

Results

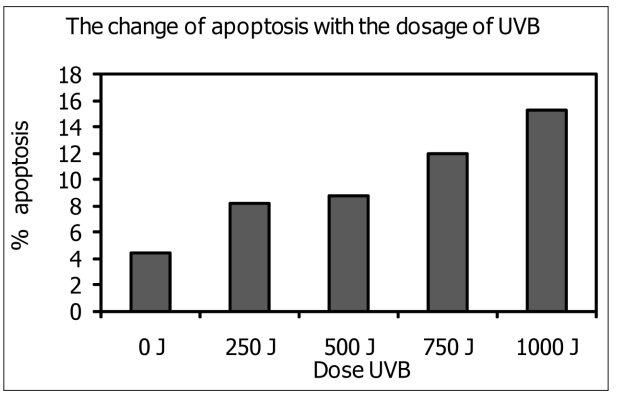


Figure 1. The effect of UVB dosage on apoptosis. Primary mouse fibroblasts were irradiated at the indicated dosages and then harvested 19 hours post UVB radiation.

Figure 1 shows the effect of UVB dosage on apoptosis. The apoptosis percentages for 0, 250, 500, 750 and 1000 J/m^2 were % 4.48, % 8.22, % 8.76, % 11.92 and % 15.24, respectively. At higher doses of UVB (data not shown) the apoptosis percentage was lower due to the increase in necrotic cell percentage. We have selected 1000 J/m^2 of UVB, a moderate dosage for mouse fibroblasts [15], in our experiments and avoided higher doses, for it would increase the necrotic cell population drastically. Figure 2 shows the forward and reverse sequences of the three point mutations generated. The sequencing primers (explained in detail in material and methods section) were used to confirm the presence of the mutations. Figure 3 shows the apoptotic response of the three AK-derived *p53*

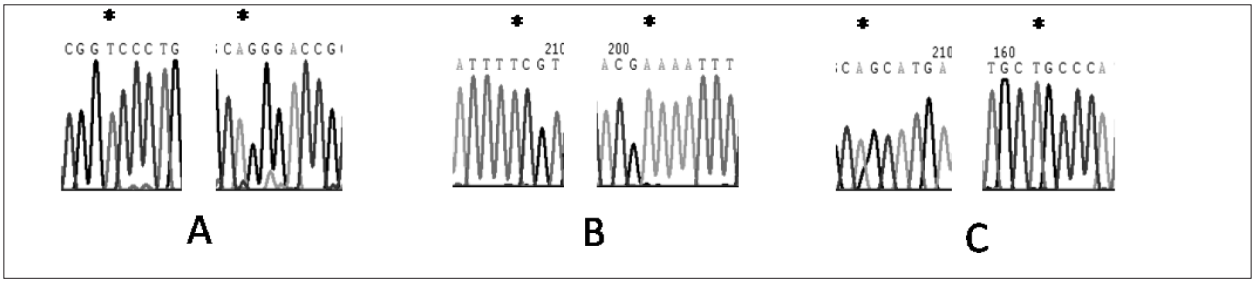
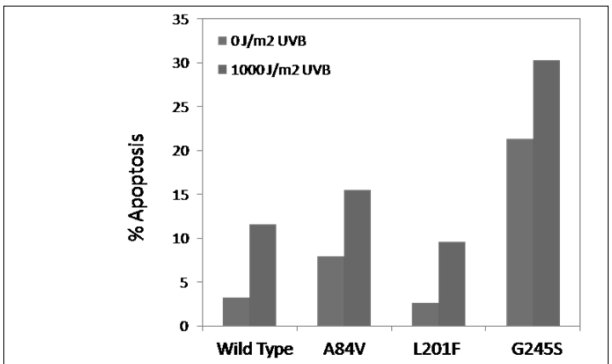


Figure 2. The sequence analysis of the mutations generated; A) A84V, B) L201F and C) G245S. For the mutation A84V the primers used were (F) 5'-CTACACCGGCGGtCCCTGCACCAGC-3' and (R) 5'-GCTGGTGCAGGGaCCGCCGGTGTAG-3'. Cytosine was replaced with Thymine (the point mutation shown in lowercase and the codon underlined in the forward primer sequence), changing the amino acid alanine to valine. For the mutation L201F the primers used were (F) 5' –GAGTGGAAGGAAATTTtCGTGTGGAGTATTTGG-3' and (R) 5' –CCAAATACTCCACACGaAAATTTCCTTCCACTC-3'. Guanine was converted to Tymine, which changes the codon from leucine to phenylalanine. For the mutation G245S the primers used were (F) 5' –CCTGCATGGGCaGCATGAACCGGAG-3' and (R) 5' –CTCCGGTTCATGCtGCCCATGCAGG-3'. In this case Guanine was converted to Adenine, which changes the amino acid glycine to serine.

mutants and the wild type. The quantified apoptosis percentage for each sample is listed in Figure 2 as a table. The results given are the average values of 4 experiments. The response of WT P53 is shown on the left. Mutation L201F resembled the wild type P53 vector, both before and after UVB treatment. Mutation A84V induced slightly elevated spontaneous apoptosis. Its UV-induced apoptosis is slightly increased as well. This region retains transcriptional activity in yeast [14]. In contrast, AK mutation that shows no transcriptional activity in yeast and is a skin tumor hotspot [8] (G245S) induced exceedingly high spontaneous apoptosis, similar to the maximum level of UV-induced apoptosis in untransfected cells. Little additional apoptosis was induced by UV; it is conceivable that the apoptotic signaling pathways for UV [16] have been activated but its manifestation is limited by other requirements for apoptosis such as DNA replication [17, 18].



	WT P53	A84V	L201F	G245S
0 J/m² UVB	3.2 ± 1.3	7.9 ±1.3	2.6 ±0.7	21.3±2.0
1000 J/m² UVB	11.6±2.0	15.5±2.4	9.6 ±1.4	30.3±1.9

Figure 3. The graph showing the response of the apoptosis percentage of wild type and mutant P53 vectors before and after UVB radiation. The results given are the average of four experiments. The table below the graph showing the apoptosis percentage values of wild type and mutant P53 vectors before and after UVB radiation, contains the average and mean value calculations of these experiments.

Discussion

Three actinic keratosis mutations were generated in this work; A84V, L201F and G245S. After generating these mutations, the protein acquires different properties, both chemically and physically. Codon 84 is situated in the proline rich domain of the protein. In mutation A84V the chemical property of the amino acid doesn't change. Alanine is converted to valine, the chemical property of the codon remains nonpolar. The elevation of the spontaneous apoptotic percentage after the mutation is generated in codon 84 (% 7.9 versus % 3.2) may be a response for protecting the proline rich domain of the protein by accumulating the mutation. After treatment with UVB, UV-induced apoptosis is still present, slightly higher than the WT scores. In G245S, the mutation in the amino acid changes the codon from glycine to serine. In that case the chemical property becomes polar where normally it is nonpolar. G245S is a hotspot mutation for non-melanoma skin cancers and is situated in the evolutionarily conserved region of the protein. Both the chemical change occurring in the codon and the position of the codon may be the reason why this mutation shows such a drastic effect on apoptosis; with a huge increase in spontaneous apoptosis and a very little additional apoptosis response after UVB treatment. L201F is found on an evolutionarily non-conserved region. On the other hand it has an important change both chemically and physically. L201F, leucine to phenylalanine, becomes an aromatic amino acid while normally it is linear. The structural change is very dramatic in this mutation. But we have seen that this mutation have no visible effect on apoptosis after UV treatment and it acts like wild type P53. The explanation for this situation can be that this mutation is not situated on an evolutionary conserved region or is not on a very functionally important region of the active folded protein. Although the mutation is

creating important changes (both chemically and physically) it has a little effect on apoptosis. The codon 201 is situated on the DNA binding region of the protein, but in the folded active structure it can be seen that it is hidden in a hydrophobic region of the protein, far away from the DNA. That may be the reason why the mutation didn't give a remarkable response to UV-induced apoptosis. Based on our data, G245S is the AK mutation that can progress to cancer *in vivo*. The frequency of an AK mutation's representation in non-melanoma skin tumors thus correlated with the extent of reduced transcriptional activity and the elevated apoptosis, with the latter occurring either spontaneously or after UV exposure. AK that progress to cancer may be those containing *p53* mutations that confer high sustained apoptosis in actinic keratoses *in vivo*, whether spontaneously or during chronic UV exposure.

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