ORIGINAL RESEARCH

The relationship between cervical human papillomavirus infection and apoptosis

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Abstract

Purpose: Cervical carcinoma is the second most common cancer among women worldwide. Viral infections, especially human papillomavirus (HPV) infections, are important factors in its etiology. Changes in apoptotic regulation are considered to have an important role in the carcinogenesis development. In this study, the relationship between apoptosis and HPV infection was investigated.

Methods: HPV DNA and HPV DNA type 16 positivity were detected in 110 cervical smear samples with Real Time PCR and sequencing was performed for HPV DNA type 18. The presence of apoptosis was investigated using TUNEL and Annexin V staining methods and analyzed by fluorescence microscope and flow cytometry.

Results: HPV DNA type 16 was detected in 9 samples (8.1%), HPV DNA type 18 positive in 6 samples (5.4%) and HPV types other than HPV type 16 and HPV type 18 in 9 samples (8.1%). A decrease apoptosis was found in HPV DNA positive samples compared with controls ($P<0.05$).

Conclusion: The decrease of apoptosis during HPV infection might cause cellular immortality and then malignant transformation.

Cervical cancer is the second most common cancer among women worldwide. Squamous cell carcinoma of the cervix often develops from preinvasive lesions called cervical intraepithelial neoplasia (CIN). It is

known that there is an association between cervical cancers, CIN development and human papillomavirus (HPV) infection.^{1,2} HPV DNA has been detected in more than 95 % cervical squamous cell carcinomas.^{3,4} More than 80 types of HPV have been isolated from different tissues and more than 20 of them are associated with cervical neoplasm.⁵ Certain HPV types such as HPV 16 and HPV 18 have been identified as an etiological factor in 90 % of cervical cancers.⁶ E6 and E7 oncoproteins of HPV type 16 and type 18 which are potentially high risk oncogenic cause cellular immortalization and consequently malignant transformation by interacting with the regulatory proteins of host cell.⁷

Apoptosis is genetically controlled death which safely enables the elimination of the cells that have completed their biological functions or have been damaged.⁸ Apoptosis is an energy based event which is induced by various signals such as UV, chemotherapeutic drugs, deficiency of developmental factor and hypoxia and which requires the synthesis of a series of proteins.¹

Changes in apoptotic pathways and regulation have important roles in carcinogenesis development.⁴ Apoptosis of damaged cells is a physiological anti-

neoplastic mechanism preventing the development of cancer because the proliferation of damaged cells leads to mutation and cancer development. There should be a balance between cell proliferation and cell destruction for a normal cell homeostasis. When this balance is upset, there would be an abnormal increase in the number of cells. The inconvenient decrease in the rate of apoptosis prolongs the life span of abnormal cells. Abnormalities in the control of apoptosis may be an important factor both in tumour progression and resistance to radiotherapy and chemotherapy.⁹

Since turnover in normal cervical epithelial cells is very rapid, the time of transition in dysplastic cells becomes shorter and the self elimination ability of cervical tumour cells is lost.¹⁰ It is possible to have an imbalance between proliferation and apoptosis compared normal cervical epithelium during cervical carcinogenesis.^{3,11}

HPV types 16 (+) and 18 (+) cervical carcinoma cell lines are quite resistant to CD95 mediated apoptosis and that the resistance to apoptotic signals is an important immunological escape mechanism during the carcinogenesis caused by viruses⁽¹⁾. The modulation of apoptosis and apoptotic regulatory proteins in high risk HPV infection is considered to have an important role in the development of cervical cancer⁽¹²⁾.

Description of changes in tumour associated cells involved in the regulation of apoptosis makes it possible to have alterations in programmed cell death during cervical carcinogenesis. There are changes in the expressions of apoptosis associated proteins in some different cervical neoplasm types.¹³

Thus, the discovery of changes in apoptosis in cells during HPV infection, considered to be an important etiological factor for the development of cervical cancer, make it possible both to understand cancer biology and to improve new methods for apoptosis treatment protocols. Furthermore, determining the apoptotic index will be an important factor in the estimation of the response to the treatment of cervical carcinoma.¹⁴

In our study, to be able to determine the association between apoptosis and HPV infection, we first aimed to determine the presence of HPV DNA and HPV DNA type 16 in cervical smear samples using Real Time Polymerase Chain Reaction method (Real time PCR) and then DNA chain analysis was performed to identify HPV type 18. Afterwards, the presence of apoptosis was investigated in healthy controls and in samples infected by HPV. Thus, we could clarify whether there was a relationship between apoptosis and HPV DNA positivity.

Methods

Ethics Review of the Proposal and the consent

The research proposal was approved by the ethical review board of the Faculty of Medicine, Gazi University. Verbal patient consent was obtained before sample collection

Patients

Smear samples obtained from 110 patients during gynecological examination using sterilized swap were taken immediately to our laboratory in sterilized phosphate buffered saline (PBS) and they were studied without any delay. The ages of the patients from whom the samples were obtained were between 23-70 (mean 49.5). Age groups were formed within 10 years. The control group comprised patients of the same age without cervical lesions and with normal cervical smear samples. All samples were first analyzed for HPV DNA by Real Time PCR and, then, the samples with HPV DNA and HPV DNA type 16 positive were sequenced and HPV DNA type 16 positivity was confirmed and type 18 positive samples were identified. After, the samples were divided into four groups and studied for apoptosis:

1. Group: HPV type 18 positive (n=6)
2. Group: HPV type 16 positive (n=9)

3. Group: HPV DNA positive and HPV types 16/18 negative (n=9)
4. Group: HPV DNA negative (control group) (n=9)

Real - time PCR analysis and DNA sequencing analysis for HPV genome

a. DNA Extraction: DNA extraction was accomplished according to kit's manual (Qiagen, DNA mini kit, Germany).

b. DNA Amplification: L1 region of the samples were replicated by using MY09/MY11 primers. Nested real-time PCR method was used for the analysis of HPV DNA and HPV16 positivity. Real time nested amplifications of MY09/11 products were done by GP5+/GP6+ primers and Cyanine-5 labeled HPV 16 DNA specific probe. Real time PCR product analysis was done by melting curve analysis on LightCycler Software version 3.5.3 (LC 2.0 Roche Diagnostics, Germany). Melting peaks of 78-82°C showed the detection of HPV DNA in the sample. Probe melting peaks of positive samples has been analyzed in the same run and HPV16 positive samples gave peaks around 68°C.

c. Sequencing: HPV 16 negative MY09/11 amplicon were sequenced by OpenGene® automated DNA sequencing system and similarity percentage of sequences were calculated by GeneObjects® software (Visible Genetics, Canada). Cycle sequencing reactions were done by using Cy5.5 dye terminator sequencing kit (Amersham Biosciences, USA). All samples were first analyzed for HPV DNA by Real Time PCR and then the samples with HPV DNA and HPV DNA type 16 positive were sequenced and HPV DNA type 16 positivity was confirmed and type 18 positive samples were identified.

Assessment of apoptosis

Programmed cell death (apoptosis) was measured using two different methods for all samples.

a. TUNEL technique for determination of apoptosis

TUNEL technique was determined using In situ Cell Death Detection Kit (Roche, Germany). The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis. Cleavage of genomic DNA during apoptosis may produce double-stranded, low molecular weight DNA fragments. These DNA strand breaks can be determined by labelling free 3'-OH termini in an enzymatic reaction. Two different methods were used for analysis of TUNEL techniques:

a.1. Analysis of DNA fragments in TUNEL techniques by fluorescence microscopy

Analysis of DNA fragments by fluorescence microscopy in samples was determined by *in situ* Cell Death Detection Kit according to the manufacturer's instructions. Cervical smear samples were dried to the air. Air dried cell samples were fixed with a freshly prepared Fixation solution (4% Paraformaldehyde in PBS) for 1 hour at room temperature. Then, cells were incubated in Permeabilisation solution for 2 minutes on ice. At the end of the incubation, TUNEL reaction mixture containing Terminal deoxynucleotidyl transferase (TdT) (enzyme solution) and nucleotide mixture (Label solution) was added and incubated for 1 hour at room temperature. TdT provides incorporation of labeled nucleotides to DNA strand breaks. Fixed and permeabilized cells in Label solution were used as negative control. Fixed and permeabilized cells with DNase I, grade I (Roche, Germany) were used as positive control. After incubation, samples were evaluated under a fluorescence microscope (Olympus, USA). The cells were counted with two different counters. The apoptotic index (AI=percentage of apoptotic cells) was determined on at least 400 cells at multiple randomly selected fields per sample.¹⁴

TABLE 1. HPV status and histological grade of the samples

	HPV Status n (%)	Histological grade (n)			
		Normal cervical epithelium	Abnormal pap smear (ASCUS)	CINII	CINIII
HPV type 18 positive	6 (5.4%)	3	-	2	1
HPV type 16 positive	9 (8.1%)	2	4	1	2
HPV DNA positive and HPV types 16/18 negative	9 (8.1%)	7	2	-	-

Apoptotic index (AI)= (number of TUNEL reactive nuclei / total number of cells counted)x100.

a.2. Analysis of DNA fragments in TUNEL techniques by flow cytometry

Cervical smear samples in PBS were prepared with a similar procedure performed by fluorescence microscope. After labeling the apoptotic cells, they were analyzed by double laser Coulter FC500 flow cytometer. Cells without label solution and terminal transferase were used as autofluorescence control of the cells

while the cells incubated only by label solution were used as negative control.

b. Assessment of apoptosis by Annexin V staining

Annexin V-FITC kit (Biosource,USA) was used. Cervical cells were washed twice in PBS and resuspended in staining buffer (FBS %1 in PBS). After centrifugation, cells were resuspended in annexin binding buffer and incubated for 10 minutes at room temperature in the dark with FITC-conjugated anti-annexin V antibody and propidium iodide (PI). Cells were washed

FIGURE 1. Fluorescence microscopic analysis of TUNEL technique showing the presence of apoptosis. *Differences from controls: $P<0.05$.

FIGURE 2. Analysis with flow cytometry for the presence of apoptosis by TUNEL technique. *Differences from controls: $P<0.05$.

FIGURE 3. A. Flow cytometry for apoptosis in the control samples by TUNEL technique (19.1%). B. Flow cytometry showing apoptosis in the samples with HPV type 16 (+) by TUNEL technique (4%)

and resuspended in binding buffer. Flow cytometric analysis was performed within 30 minutes of staining (Coulter FC500, USA).

Statistics

Results were analysed using one-way analysis of variance (ANOVA). The Bonferroni test was used as Post Hoc analysis. $P < 0.05$ was considered statistically significant.

Results

HPV DNA positivity

Real Time PCR analysis of cervical smear samples obtained from 110 patients during gynecological examination revealed 24 samples HPV DNA positive (21.7%) (Table 1).

Samples in the control group not infected by HPV were reported as normal cervical smears.

The presence of apoptosis

Of the HPV type 16 and type 18-confirmed samples, we evaluated the presence of apoptosis.

a. TUNEL technique

a.1. Fluorescence microscopic analysis of TUNEL technique

No difference was observed between samples with HPV type 16 (+) and HPV type 18 (+) and the samples infected by HPV types other than types 16 and 18. However, there was a difference between the samples with HPV type 16 positive and HPV type 18 positive and the control samples ($P < 0.05$). No difference was observed between the samples with HPV type 16 positive and type 18 positive (Fig. 1).

a.2. Flow cytometric analysis of TUNEL technique

This revealed a difference between all the samples HPV DNA (+) and the control group. There was also a difference between the samples with HPV 16 type (+) and HPV 18 type (+) and the samples infected by HPV types other than types 16 and 18 ($P = 0.005$, $P = 0.001$ respectively). However, no difference was observed between the samples with HPV type 16 (+) and type 18 (+) in respect to (Fig. 2). The flow cytometric images of a sample with HPV type 16 (+) and another sample from the control group are given in Figure 3.

FIGURE 4. Flow cytometric analysis by Annexin V staining method for apoptosis. *Differences from controls: $P < 0.05$.

b. Annexin V staining method

There was a decrease in the presence of apoptosis in HPV DNA (+) samples compared with the control group. Similarly, a decrease in the presence of apoptosis was observed in the samples with HPV type 16 (+) and HPV type 18 (+) compared with both samples infected by HPV types other than types 16 and 18 ($P = 0.009$, $P = 0.023$ respectively) and the control group ($P < 0.05$) (Fig. 4).

In our study, the presence of apoptosis in HPV DNA positive cervical smear samples with CIN II and CIN III had an inverse correlation with the advanced dysplasia histologically and apoptosis decreased in the samples with increased dysplasia.

Discussion

Apoptosis is considered to play an important role in viral pathogenesis. The replication time of persistent

DNA viruses that proliferate slowly is long and the cell may die because of apoptosis before the completion of replication. For this reason, almost all of these viruses encode antiapoptotic gene products and thus the persistent and latent viruses may keep the cell they infected alive.¹⁵ DNA viruses, such as HPV, have developed some mechanisms that will prevent apoptosis by inhibiting the tumor suppressor function of p53 with the mediator E6 and E7 proteins.¹⁶ The relationship between HPV infection and apoptosis is disputable and has not been clarified. While in some studies such association has been reported, in others there is no association between the presence of HPV DNA and apoptosis.¹⁷ For this reason, our study is important because we used three different methods, to determine especially the high risk HPV DNA presence and apoptosis relationship in cervical samples.

We observed that apoptosis decreased more in samples infected by high risk HPV such as HPV types 16 and 18. We also found out that apoptosis decreased remarkably in the cervical samples infected by HPV types 16 and 18 which especially showed advanced phase dysplastic changes. This suggests that the lessened sensitivity to apoptosis may be an important factor in the cervical carcinogenesis associated with HPV infection. However, in our study, since only 3 of the 24 samples with HPV DNA positive had CIN III, the sample size was insufficient to explain apoptosis-neoplasia associations. We believe that future studies will support our proposition.

Our results are consistent with others.^{5,19,20} Ahmed et al⁵ determined the positive relationship between cervical cancer and HPV infection and, as in our study, they indicated that the apoptotic index is higher in HPV type 16 negative samples compared with HPV type 16 positive samples. Thus, HPV may have a role in the inhibition of apoptosis. These findings support our idea that the apoptotic index is lower in the samples with HPV type 16 and HPV type 18 positive than it is in other HPV positive types. Thus, inhibition caused by high risk HPV infection in apoptosis, may explain the effects of HPV type 16 and HPV type 18,

which are important factors in the development of cervical cancer, during oncogenesis. HPV type 16 and HPV type 18 have antiapoptotic effect on the viral E6 oncogene.⁶ Nair et al¹⁹ remarked that high risk HPV infection occurs with decreased apoptosis. The change in apoptotic regulatory proteins and apoptosis during high risk HPV infection may be important in the development of cervical cancer.

There are many studies of gene expression of proteins regulating apoptosis to determine the association between apoptosis and HPV infection. Our study is different in that it helps to determine the presence of apoptosis directly in cervical samples and aims to find the most suitable method by applying three methods to detect apoptosis. Our results are consistent with those of flow cytometric analysis of TUNEL technique and o Annexin V staining. Only the results obtained by fluorescence microscope differ, in part, from that obtained by other methods. The reason that no difference was observed by fluorescent microscope may be that the fluorescent microscope is not an ideal tool to identify detailed cell structures.¹⁸ Thus, in our study, using flow cytometry analysis to detect apoptosis was considered as a more convenient method.

Several studies have reported that expression of most of the apoptotic proteins inhibiting apoptosis increases in cervical cancer.^{9,13} Since a positive relationship between HPV infection and the development of cervical cancer has been observed, it is not surprising that apoptosis decreases in cervical samples with HPV infection. Our data will encourage the development of clinical applications to determine the risk of cervical cancer progression, in patients infected by HPV where apoptotic products are used as a marker, with new immunotherapeutic approaches, and of new vaccine targets.

In conclusion, apoptosis decreased during high risk HPV infection. We suggest that there is a relationship between HPV infection and apoptosis, and that HPV-mediated changes in the mechanisms of apoptotic regulation may have important effects in the development of cervical carcinoma. Moreover,

apoptosis-related proteins may be used together with HPV status as a marker in determining the prognosis of cervical lesions. More complete understanding of these mechanisms will help to detect the cancer risk and to develop protective and therapeutic methods.

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