Correlation of apoptosis with tumour cell differentiation, progression, and HPV infection in cervical carcinoma

Y Shoji, M Saegusa, Y Takano, M Ohbu, I Okayasu

Abstract

Aims—To clarify the significance of apoptosis in the progression of uterine cervical neoplasias, including cervical intraepithelial neoplasia (CIN), microinvasive carcinoma (MIC), and invasive squamous cell carcinoma (ISCC) categories, in relation to cell proliferation and human papilloma virus (HPV) infection.

Methods—Forty six cases of CIN I/II, 75 of CIN III, 16 of MIC, and 44 of ISCC were examined using formalin fixed and paraffin wax embedded samples. The TdT mediated dUTP-biotin nick end labelling (TUNEL) method for detection of apoptotic cells was performed along with Ki-67 immunohistochemistry. Presence of HPV-DNA was confirmed by PCR-RFLP assay. **Results**—Apoptotic labelling indices, calculated after counting positive nuclei among at least 2000 nuclei, showed significant positive correlation with histological malignant grading in CIN and tumour cell invasion into stroma. In contrast, similar Ki-67 labelling index values were found in CIN, MIC, and ISCC. Although HPV-DNA was detected in 35/46 CIN I/II (76.1%), 53/74 CIN III (71.6%), 9/16 MIC (56.3%), and 36/44 ISCC (81.8%), there was no apparent relation with the apoptotic labelling indices.

Conclusions—Apoptosis in cervical neoplasias may be closely related to tumour cell differentiation and progression. It also seems unlikely that HPV itself is directly related to pathways regulating apoptosis.

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Keywords: apoptosis, HPV, CIN, SCC, Ki-67

Although a relation between genesis of cervical carcinomas and human papillomavirus (HPV) infection has been considered, it is widely accepted that HPV infection alone is not responsible for cervical neoplasia, and several cofactors, such as other infectious agents, genetic abnormalities, and smoking have been identified from epidemiological and experimental investigations.¹ The fact that spontaneous regression frequently occurs in cervical intraepithelial neoplasia (CIN) type I or II lesions but a part of CIN III progresses to invasive carcinoma^{2,3} suggests that selection of a cell population with high malignant potential may be closely linked to progression of cervical neoplasias.

The rate of cell accumulation is determined by the balance of cell proliferation and death. Apoptosis (programmed cell death) is an important mechanism of cell loss in tissue modelling for vertebrate development, with apparent differences from necrosis.⁴ For example, apoptotic cells are not associated with an inflammatory reaction, even when present in large number.⁵ Regulation of the process leading to death involves expression of several oncogenes or oncosuppressor genes, such as *c-myc*,⁶ *p53*,⁷ and *bcl-2*.⁸ Recently, the significance of apoptosis in development or growth of human malignant tumours has become a major topic of discussion, particularly in relation to cancer treatment by irradiation and anticancer drugs.⁹

The TdT mediated dUTP-biotin nick end labelling (TUNEL) method developed by Gavrieli *et al*¹⁰ is able to detect apoptosis at the single cell level. In the present study, we assessed the significance of apoptosis for progression of cervical neoplasias—including CIN, microinvasive carcinoma (MIC), and invasive squamous cell carcinoma (ISCC)—by this approach, as well as using Ki-67 immunohistochemistry to estimate cell proliferation. In addition, the relation between apoptosis and HPV infection was also examined using a polymerase chain reaction (PCR) assay for detection of HPV-DNA.

Methods

CASE SELECTION

A total of 181 cases was selected from the surgical patient's files of Kitasato University Hospital. Tissue slices had been fixed in 10% formalin and embedded in paraffin wax. Histological examination was mainly performed according to Richart's criteria¹¹; thus cervical intraepithelial neoplasia (CIN) I encompasses mild dysplasia, CIN II, moderate dysplasia, and CIN III, severe dysplasia and carcinoma *in situ* (CIS). The cases investigated were divided into 46 cases of CIN I and II, 75 cases of CIN III, 16 cases of MIC, and 44 cases of ISCC.

TUNEL ASSAY

TUNEL procedure was performed according to the description of Gavrieli *et al*,¹⁰ with minor modification. Briefly, after routine deparaffinisation and treatment with 0.3 % hydrogen peroxidase (H₂O₂), sections were digested with 100 µg/ml proteinase K (Merck) at room temperature for 15 minutes and washed with distilled water. Sections were immersed in TDT

Department of Pathology, Kitasato University School of Medicine, 1-15-1 Sagami-hara, Kanagawa 228, Japan
Y Shoji
M Saegusa
Y Takano
M Ohbu
I Okayasu

Correspondence to: Makoto Saegusa MD.

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Figure 1 Note apoptotic cells (indicated by arrows) showing condensation of nuclear chromatin and loss of cell-cell contacts in haematoxylin-eosin sections. (A) CIN II, $\times 345$; (B) ISCC, $\times 345$.

buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) at roomtemperature for 10 minutes, and then incubated with 0.5 unit/ μ l terminal deoxynucleotidyl transferase (Boehringer Mannheim/Yamanouchi) and 0.01 nmol/ μ l biotinylated-11-dUTP (Sigma) in TDT buffer at 37°C for 60 minutes. Sections were rinsed with distilled water and the routine avidin-biotin-peroxidase complex method (Vecstastain ABC kit, Vector Laboratories) was used for detection of apoptotic signals.

Table 1 Correlation between malignant grading and apoptotic and Ki-67 labelling indices. Values are means (SD)

	Apoptotic LI	Ki-67 LI
CIN I/II (n=46)	0.26 (0.56) ^a	47.20 (14.45)
CIN III (n=74)	0.82 (1.43) ^{a,b}	46.67 (15.97)
MIC (n=16)	1.81 (1.58) ^{b,c}	43.32 (13.81)
ISCC (n=44)	4.65 (2.95) ^c	49.31 (14.00)

LI=labelling index; CIN=cervical intraepithelial neoplasia; MIC=microinvasive carcinoma; ISCC=invasive squamous cell carcinoma.

^{a,b} $p < 0.005$.

^c $p < 0.001$.

IMMUNOHISTOCHEMISTRY

Ki-67 immunoreactivity was detected by a combination of microwave oven heating and the standard Streptavidin-biotin-peroxidase complex method (Histofine SAB-PO (M) kit, Nichirei Co), using rabbit anti-human Ki-67 antigen ($\times 300$ dilution, Dako).

DNA EXTRACTION AND POLYMERASE CHAIN REACTION (PCR) ASSAY

For detection of HPV-DNA, DNA samples were extracted from several serial 10 μ m thick paraffin wax sections, through phenol/chloroform treatment as described previously. The quality of the DNAs extracted was confirmed with β globin gene specific primers which was able to amplify the 355 base pair fragment.¹²

The PCR assay was performed using the consensus primers for HPV L1 region, L1C1 and L1C2, which is able to detect 0.001 pg of DNA of HPV types 6, 11, 16, 18, 31, 33, and 52, and 0.1 pg of those of HPV types 42 and 58.¹³ Ten microlitres of PCR mixture containing 10 ng template DNA, 1 μ M of each primer, and 0.5 unit Taq DNA polymerase (Takara) were prepared, and samples were amplified through 45 cycles. PCR was carried out as a replicate or triplicate assay and water instead of DNAs was used as a negative control.

HPV subtyping was determined by the restriction fragment length polymorphism (RFLP) assay with *Rsa* I, *Dde* I, and *Hae* III.

APOPTOTIC AND KI-67 LABELLING INDICES

The slide was randomly moved and five fields were selected for each case using a $\times 40$ objective and $\times 10$ ocular. All TUNEL signal positive or Ki-67 immunolabelling nuclei from among at least 2000 tumour cells were then counted. In CIN lesions, these counting procedures were performed in the whole epithelial layers. Apoptotic and Ki-67 labelling indices (A-LI and Ki-67 LI) were calculated as number per 100 cells.

STATISTICS

Values are given as mean (SD). Statistical analysis was performed using the Student *t* test.

Results

APOPTOSIS IN CERVICAL NEOPLASIAS

Cells undergoing apoptosis showed condensation of nuclear chromatin, nuclear fragments (apoptotic bodies), and loss of cell-cell contacts in conventionally haematoxylin-eosin (H and E) sections (fig 1). These changes were sometimes not associated with necrotic or inflammatory foci.

TUNEL signals were detected not only in these cells but also in morphologically viable cells at the start of apoptosis, as identified by distinct nuclear staining. Positive cells were sporadically found in CIN and appeared in clusters in ISCC (fig 2). However, comparative observation of H and E stained serial sections

Figure 2 Cells undergoing apoptosis (indicated by arrows) show a distinct positive nuclear staining by the TUNEL method. (A) CIN II, $\times 345$; (B) ISCC, $\times 345$.

was necessary for verification, as non-specific staining of necrotic foci and inflammatory cells was evident. In addition, a few cells located at the most superficial layer of the normal cervical epithelia (edge of sections) were occasionally showed not only nuclear but not cytoplasmic staining, and it was difficult to determine whether these signals were specific or non-specific.

Data on the correlation between apoptosis and histopathological grading of cervical neoplasias are summarised in table 1. The A-LI clearly increased together with tumour progression, this being statistically significant.

CELL PROLIFERATIVE ACTIVITY IN CERVICAL NEOPLASIAS

In CIN, Ki-67 immunoreactivity was predominantly found in basaloid cells (basal and parabasal cells). Koilocytotic cells in the upper layers also occasionally showed positive staining. A heterogeneous distribution of Ki-67 labelling cells within individual ISCC was noted (fig 3).

As evident from the results in tables 1 and 2, there was no clear correlation between Ki-67 LI and morphological malignant grading.

RELATION BETWEEN APOPTOSIS AND HPV INFECTION

With consensus primers for the HPV L1 region, amplified PCR products (approximately 260 base pairs (bp)) were detected, as shown in fig 4. HPV types were determined by RFLP analysis. For example, the PCR product originating from HPV 16 was cleaved into 169 bp and 84 bp fragments by *Dde* I digestion and was not digested by *Rsa* I.

PCR and RFLP analysis revealed HPV-DNA in 35/46 CIN I/II (76.1%), 53/74 CIN III (71.6%), 9/16 MIC (56.3%), and 36/44 ISCC (81.8%). Of the HPV-DNA positive cases, HPV 16/18 was found in 28 (80%) in CIN I/II, 38 (71.7%) in CIN III, eight (88.9%) in MIC, and 31 (86.1%) ISCC.

No apparent relation between A-LI and the presence of HPV-DNA could be demonstrated (table 2).

Discussion

The TUNEL technique is based on the specific binding of TdT to 3'-OH of DNA fragments in apoptotic nuclei, permitting the in situ visualisation of apoptotic cells.¹⁰ However, it is necessary to be careful when evaluating results as necrotic cells and some inflammatory cells, including neutrophils and lymphocytes, are also stained.

Table 2 Correlation between HPV infection and apoptotic and Ki-67 labelling indices. Values are means (SD)

	Apoptotic LI		Ki-67 LI	
	HPV (+)	HPV (-)	HPV (+)	HPV (-)
CIN I/II	0.20 (0.43) ^a (n=35)	0.45 (0.85) (n=11)	49.51 (14.59) (n=35)	39.86 (11.74) (n=11)
CIN III	0.98 (1.62) ^{ab} (n=53)	0.41 (0.67) (n=21)	48.21 (15.37) (n=53)	43.11 (17.10) (n=21)
MIC	2.20 (1.84) ^{bc} (n=9)	1.31 (1.09) (n=7)	41.14 (12.8) (n=9)	46.11 (15.54) (n=7)
ISCC	5.15 (2.92) ^{cd} (n=36)	2.39 (1.97) ^d (n=8)	47.46 (13.65) (n=36)	57.39 (13.43) (n=8)

LI=labelling index; CIN=cervical intraepithelial neoplasia; MIC=microinvasive carcinoma; ISCC=invasive squamous cell carcinoma.

^ap<0.01.

^bp<0.05.

^cp<0.01.

^dp<0.05.

Figure 3 Ki-67 immunohistochemistry. (A) Note the strong Ki-67 immunoreactivity of koilocytotic cells as well as basal and parabasal cells in CIN II; $\times 345$. (B) Heterogeneity of Ki-67 immunoreactivity in ISCC. The tumour nest on the right side shows a relatively high immunolabelling as compared with that on lower left side; $\times 173$.

Our study showed that apoptosis clearly increases with malignant grading in uterine cer-

vical neoplasias. As a basic principle, CIN grading is positively correlated with abnormal maturation of basaloid cells. Morphological change of tumour cells in the process of development from CIN to MIC or ISCC may be considered as atypical differentiation as it has been asserted that one indication of squamous cell maturation is an increase in cytoplasm.¹⁴ In the HL-60, human promyelocytic leukaemia cell line, terminal differentiation induced by retinoic acid treatment serves to facilitate the apoptotic response.¹⁵ A positive correlation between frequency of apoptosis and histological malignant grading (loss of differentiation) has been noted in prostatic cancers¹⁶ and colonic tubular adenomas.¹⁷ It is therefore suggested that abnormal differentiating features may play an important role in the susceptibility of tumour cells to apoptosis in cervical neoplasias. In addition, our investigation revealed a statistically significant difference between MIC and ISCC, suggesting that the frequency of apoptosis may be linked with tumour progression. There have been similar findings in human gastric carcinomas.¹⁸

A close association between apoptotic and mitotic indices has been found in various human tumours.^{16,17} In the present study, we used Ki-67 immunohistochemistry to determine the cell proliferative activity instead of estimating the mitotic index. Earlier studies showed that Ki-67 immunoreactivity can be detected in all phases of the cell cycle except G₀.^{19,20} Results for this index are closely related to those for several prognostic indices in non-Hodgkin lymphomas²¹ and breast carcinomas.²² However, Shepherd *et al*²³ have reported that in colorectal carcinomas Ki-67 labelling rates do not correlate with known prognostic indices such as tumour differentiation, Dukes' stage, or lymph node metastasis, suggesting that the proliferative status alone has no influence on the prognosis after surgical treatment. Our results also show no apparent relation between Ki-67 LI and either A-LI or morphological malignant grading in

Figure 4 HPV types 16, 18, 52, and 58 demonstrated by PCR-RFLP assay: lane a, uncut; lane b, Dde I digestion; lane c, Rsa I digestion. m, molecular markers (DNA molecular weight markers V, Boehringer Mannheim/Yamanouchi).

cervical neoplasias. This lack of correlation may be due to the marked variation in Ki-67 positive cell distribution within individual tumours, or the detection of an extensive cell cycle phase by Ki-67 immunohistochemistry. The fact that koilocytotic cells existing in upper layers in CIN II also occasionally reacted to Ki-67 as well as the basaloid cells resulted in a high labelling index value.

Previous studies have shown that HPV can play a central role in the cervical tumorigenesis,^{24,25} and that the overall HPV prevalence in cervical carcinomas was 92.9% and ranged from 75% to 100% by country.²⁶ The oncoproteins, E6 and E7, produced by HPV, are capable of binding to p53 and the retinoblastoma susceptibility gene product Rb, respectively.^{27,28} Wild-type p53 can accelerate the induction of apoptosis,⁷ in contrast to Rb itself which can inhibit apoptosis in certain circumstances.^{29,30} Moreover, complexes of E7 and Rb are reported to release Rb mediated repression of *c-myc*^{31,32} which stimulates apoptosis. In this context it is of interest that *c-myc* expression has been observed in invasive cervical carcinomas, closely correlating with prognosis.³³ These findings may indicate a complex linkage between HPV infection and apoptosis. In this study, no apparent correlation was found between presence of HPV-DNA and apoptosis, with the exception of the ISCC cases, suggesting that HPV itself is not directly involved in the apoptosis regulatory pathways in cervical neoplasias. This conclusion is supported by the finding of the same degree of positivity for HPV-DNA in CINs, MIC, and ISCC.

In conclusion, our study provides evidence for a close correlation between apoptosis and differentiation and progression of cervical tumour cells, independent of HPV infection.

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