한국현미경학회지 제40권 제4호 2010
Korean J. Microscopy 40(4), 229~235(2010)

Synergistic Effects of 5-Fluorouracil (FU) and Curcumin on Human Cervical Cancer Cells

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(Received November 17, 2010; Revised December 24, 2010; Accepted December 26, 2010)

5-fluorouracil과 curcumin의 복합투여에 의한 자궁암세포의 성장억제와 p53유전자 발현의 상승 효과

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ABSTRACT

Cervical cancer is associated with low antioxidant status. It has a high prevalence especially amongst woman in Asia and is a leading cause of cancer death. Cancer chemotherapy *in vivo* improved in cases with high p53 expression in the tumor tissue. The restoration of p53 levels could be a potential strategy to increase chemoresponsiveness. Under circumstances where damage is extensive, p53 plays a direct role in trigering apoptosis. To investigate the effect of curcumin (CMN) as an antioxidant agent on anticancer agent 5-fluorouracil (5FU) induced apoptosis and p53 expression, HPV-18 positive HeLa cells were treated with noncytotoxic amounts of antioxidant. Curcumin induced apoptosis in cervical cancer cells. Morphological hallmarks of apoptosis such as nuclear fragmentation and internucleosomal fragmentation of DNA were observed. CMN caused upregulation of p53 expression, evident from Western blotting data and also increased the susceptibility/apoptosis induced by 5FU. These results show that increasing drug sensitivity of cervical cancer cells by upregulation of p53 using CMN is novel approach and could have a possible therapeutic potential in cervical cancer.

Keywords : Cervical cancer, 5-Fluorouracil, Curcumin

INTRODUCTION

Cervical cancer primarily has a viral etiology and HPVs have been shown to be involved in the pathogenesis of viral, vulval, penile and perianal cancer (Zur Hausen, 1996). The current standard of care for patients with stage IIA to IVA cervical cancer is chemoradiotherapy involving 5-fluorouracil (5FU) and cisplatin (CDDP) (Morris et al., 1999). Several agents and drug combinations including carboplatin, CDDP, 5FU, ifosfamide, etoposide, and most recently taxanes have been used as radiation sensitizers (Candelaria et al., 2006). The cause for poor responsiveness to chemotherapy lies in the etiopathogenesis of cervical cancer i.e., HPV infection and loss of tumor

"This work was supported by the grant of the Korean Ministry of Education, Science and Technology" (The Regional Core Research Program/Chungbuk BIT Research-Oriented University Consortium).
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suppressor gene function due to inactivation of p53 by HPV encoded viral oncoproteins. P53 is protein critical to the maintenance of genomic integrity, particularly after genotoxic stress.

It is noteworthy that apoptosis-inducing ability seems to have become a primary factor in considering the efficiency of chemopreventive agents (Choudhuri et al., 2002). Evolving interest in recent years has focused on diet-derived agents augmenting apoptosis as possible candidates for evaluation of their synergistic efficacy in combination with chemotherapeutic agents.

Curcumin [1,7-bis-(4-Hydroxy-3-methoxyphenyl)-1,6 heptadiene-3,5-dione] is a major constituent of turmeric powder extracted from the rhizome of the plant Curcuma longa found in South and Southeast tropical Asia. Curcumin (CMN) has attracted interest (Somansundaram et al., 2002; Thangapazam et al., 2006) because of its anti-inflammatory and chemopreventive activities (Conney et al., 1991; LoTempio et al., 2005; Bisht et al., 2007) inhibiting tumor progression against skin, oral, intestinal, breast, colon and prostate cancer in animal studies (Inano et al., 1999; Kawamori et al., 1999). However the efficacy of CMN in cervical cancer cell lines has not been fully determined.

Our objective in this study was to investigate whether there is any synergism between 5FU and CMN on human cervical carcinoma, HPV-18 positive HeLa cells. We evidenced apoptosis in observation of DNA fragmentation assay and flow cytometry and cell morphology using transmission electron microscopy. Also we observed expression of p53 with Western blotting analysis to prove interaction of apoptosis induction and expression level of p53 protein.

MATERIALS AND METHODS

1. Materials

Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), streptomycin, penicillin, L-glutamine, sodium pyruvate and non-essential amino acids were purchased from Gibco BRL, Daejeon Korea. Curcumin, and general reagents were purchased from Sigma Aldrich, Seoul Korea. The remaining chemicals were purchased from local company and were of highest purity grade.

2. Cell culture

Human cervical cancer cells (HeLa) were obtained from NIH, NCI, USA. Cells were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum. 5% L-glutamate and 0.5% sodium pyruvate, antibiotics (100 μg/mL streptomycin and 50 U/mL penicillin) at 37˚C in a humidified incubator containing 5% CO_2. Cell were trypsinized and sub-cultured at 1 : 3 ratio for routine maintenance and experiments.

Cells were plated to a density of 3×10^5 cells per 100 mm plate. An equal number of plates were treated with different concentrations 0.1, 1.0, 10.0 μg/mL 5FU and/or 1.0 μg/mL CMN in dimethysulfoxide (DMSO) or DMSO alone. Cells were collected from each plate at the end of 24 hours incubation period for cell growth, cell cycle analysis. Each experiment was repeated 3 times to confirm reproducibility.

3. Transmission electron microscopy (TEM)

The cells were prepared as above, fixed in 2% formaldehyde. Then after a washing in 0.1 M PBS, they were fixed with 2.5% glutaraldehyde (0.1 M PBS, pH 7.4) treated with 1% osmium tetroxide (0.1 M PBS, pH 7.4), fixed dehydrated in ethanol, and finally embedded in epoxy resin. Thin section (60 nm thickness) were sliced, and then were double-stained with uranyl acetate and lead citrate. The section were then examined under a JEM-2000FX electron microscope (JEOL, Tokyo, Japan)

4. DNA fragmentation assay

Cells were seeded in 100 mm dishes at seeding densities of 3×10^5 and treated with different concentrations of 0.1, 1.0, 10.0 μg/mL of 5FU and/or 1.0 μg/mL of CMN for 24 hours. Cells were suspended in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, incubated with 100 μg/mL RNase A at 37˚C for 1 hour. The DNA in the solution was precipitated with ethanol at −20˚C, dissolved in 10 mM Tris-HCl and 10 mM EDTA, and eletrophoresed for 30 minutes at 100 V through 2% agarose gel containing 0.5 μg/mL ethidium bromide.

5. Flow cytometry

Cells were treated with 0.1, 1.0, 10.0 μg/mL of 5FU and/or 1.0 μg/mL of CMN for 24 hours and then harvested. Cells were fixed in 70% ethanol and left overnight at −20˚C. Cells were then washed with PBS and incubated in staining solution (20 μg/mL propidium iodide, 50 μg/mL RNase, 0.1% Triton X-100 and 0.1 mM EDTA) for 2 hours at 4˚C in dark. The DNA content of the cells was measured by flow cytometer (Becton Dickenson, FACSCalibur).

6. Western blot analysis

Cells were washed twice in PBS and lysed in lysis buffer. Protein concentrations were determined with spectrophotometer. Equal protein loading was confirmed by detection of p53 using mouse p53 primary antibody (1 : 1,000 dilutions) and goat anti-mouse secondary antibody (1 : 5,000 dilutions). The bands were analyzed and quantified using image scanner densitometer (Alpha innotech, USA). The density of control was taken as 1 and results of treatment were expressed in relation to the control as relative unit (RU).

7. Statistical analysis

Statistical analysis was performed using the Student's t-test to determine significant differences between treatment groups, with p values < 0.05 indicating statistically significant differences. Western blotting datas was expressed as mean of three individual experiments ± standard deviation (SD) and was calculated using Microsoft excel.

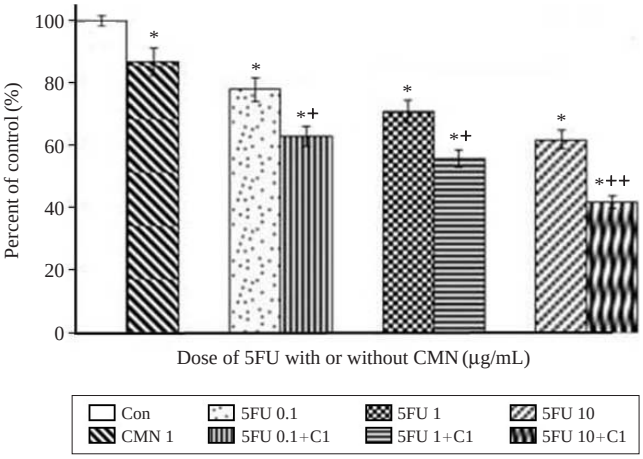

Fig. 1. 5FU-induced growth inhibition is potentiated by CMN. HeLa cells were treated with (1) 0, (2) 1 μg/mL CMN, (3) 0.1 μg/mL 5FU, (4) 0.1 μg/mL 5FU+1 μg/mL CMN, (5) 1 μg/mL 5FU, (6) 1 μg/mL 5FU+1 μg/mL CMN, (7) 10 μg/mL 5FU, (8) 10 μg/mL 5FU+1 μg/mL CMN as indicated and incubated for 24 hours, and cell viability was assessed as described in Materials and Methods. All determinations were made in triplicate. *: $p < 0.05$ compared with control, +: $p < 0.05$ compared with 5FU to 5FU+CMN. Experiments were repeated three times.

RESULTS

1. 5FU and/or CMN inhibit cell proliferation

The antiproliferative effects of 5FU and/or CMN on HeLa cervical cells was determined by cell count with tryphan blue.

Cells were exposed to varying concentrations of 5FU (0.1, 1.0, 10.0 μg/mL) and/or CMN (1.0 μg/mL) for 24 hours. In HeLa cells, the percentage of control cells at 1.0 μg/mL CMN and 0.1, 1.0, 10.0 μg/mL 5FU concentration was 87.2±4.1%, 77.8±3.2%, 71.2±2.3%, 62.4±2.7% respectively. The cell viability was reduced gradually by 5FU in a concentration-dependent manner. Whereas a combination dose of 0.1, 1.0, 10.0 μg/mL 5FU and 1.0 μg/mL CMN was 62.7±2.4%, 56.0 ±1.9%, 41.6±1.4% respectively.

The values of the combined treatment with 1.0 μg/mL CMN decreased significantly compared with 5FU alone ($p < 0.05$).

2. CMN induces morphological features of apoptosis

To check whether CMN has any role in the regulation of apoptotic morphology, cervical cancer cells were treated with 1.0 μg/mL CMN for 24 hours. In untreated cells, all the cells show normal feature (Fig. 2A, C), whereas the growth of CMN-treated cells inhibited distinctly (Fig. 2B, arrow). Phenotypically, apoptosis is characterized by cell shrinkage, chromatin compaction nuclear blebbing and collapse of the nucleus into small intact fragments which were evident from the transmission electron microscopy of 5FU and/or CMN-treated HeLa nucleus (Fig. 2D). Apoptotic bodies were observed to be comparatively more in wells treated with the combination as compared with individual treatments (data not shown).

3. CMN potentiated 5FU-induced cell damage and DNA fragmentation in HeLa cells

The degradation of DNA into multiple internucleosomal fragments of 180～200 base pairs is a distinct biochemical hallmark for apoptosis. The internucleosomal DNAase activity in apoptotic cells was studied by DNA fragmentation assay. To determine whether the CMN have any effect on 5FU-induced apoptosis in HeLa cells, we isolated DNA from the exposed cells for 24 hours to 5FU with or without CMN treatment. The apoptotic feature of a 180 bp DNA ladder was detected in CMN and 5FU treated HeLa cells (Fig. 3: Lane 2～6). A more pronounced DNA ladder was observed in the presence of 1.0 μg/mL of CMN (Fig. 3: Lane 7～9), indicating enhanced apoptosis.

4. Effect of CMN on HeLa cell cycle progression

Apoptosis was measured using flow cytometery. We asses-

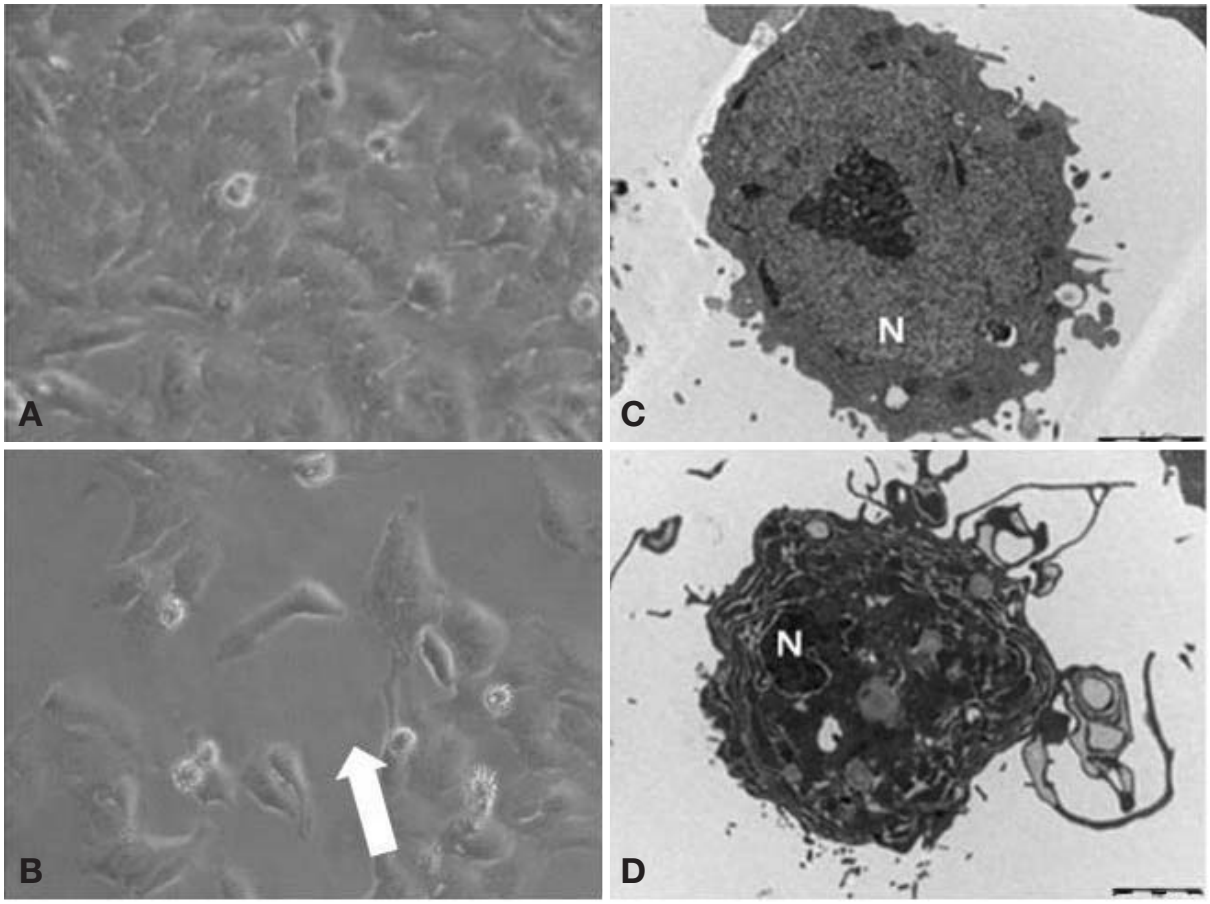

Fig. 2. Changes in morphology induced by CMN. HeLa cells were seeded in 60 mm plates and then treated 1.0 μg/mL CMN for 24 hours. Cells were viewed under inverted microscope (A, B) at a magnification ×100, and the image was captured with an attached camera (DP12) and transmission electron microscope (C, D) photographed as described under Materials and Methods. Untreated cells show unremarkable feature (A, C), whereas after 1.0 μg/mL CMN treatment for 24 hours, cells exhibit inhibition of proliferation (B, arrow) and condensation of nuclear chromatin (D). N: nuclear, Scale bars are 1 μm (C, D).

sed the effect on cell cycle progression of CMN treatment. Also, cell cycle distributions in HeLa cells treated with 5FU in combination with CMN were analyzed by flow cytometry. CMN caused growth arrrest at the G2/M phase of the cell cycle. CMN treatment with 5FU arrested HeLa cells at the G2/M phase and eventually increased susceptibility of apoptosis (sub-G0G1: A_0 region) induced by 5FU (Fig. 4D).

5. Upregulation of p53 expression

To confirm apoptosis in response to combined treatment with CMN and 5FU, we evaluated p53 protein expression by Western immunoblotting. 1 μg/mL CMN was significantly induced p53 expression compared with untreated control and 0.1 μg/mL 5FU. As shown in Fig. 5, combined treatment with curcumin and 5FU augmented 5FU-induced p53 expression. Taken together, the above results confirm that a combination of 0.1 μg/mL 5FU and 1 μg/mL CMN makes HeLa cells more susceptible to apoptosis.

DISCUSSION

The concept that cancer can be prevented or certain diet-derived substances can postpone its onset is currently eliciting considerable interest. Several plant-derived components are currently successfully employed in cancer therapy. Apoptosis plays a pivotal role in the regulation of the development and homeostasis of multicellular organisms (Steller, 1995). Balance between tumor cell proliferation and spontaneous cell death via apoptosis has an important role in the regulation of tumor cell growth (Thompson, 1995). Anticancer drugs function by

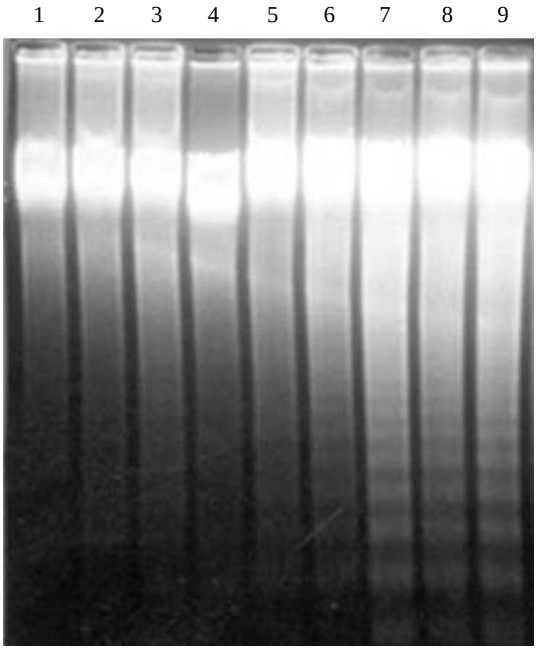

Fig. 3. Curcumin (CMN) augments 5FU-induced DNA fragmentation. HeLa grown in 100-mm dishes were treated with 0.1, 1.0, 10.0 μg/mL 5FU with and without 1.0 μg/mL curcumin for 24 hours. Cells were harvested and the oligonucleosomal DNA fragments were isolated, separated by gel electrophoresis, and analyzed as described under Materials and Methods. These results were confirmed in another independent experiment. Lane 1: control, Lane 2: 0.1 μg/mL 5FU, Lane 3: 1.0 μg/mL CMN, Lane 4: 0.1 μg/mL 5 FU, Lane 5: 1.0 μg/mL 5FU, Lane 6: 10.0 μg/mL 5FU, Lane 7: 0.1 μg/mL+1.0 CMN, Lane 8: 1.0 μg/mL 5FU+1.0 CMN, Lane 9: 10.0 μg/mL 5FU+1.0 CMN.

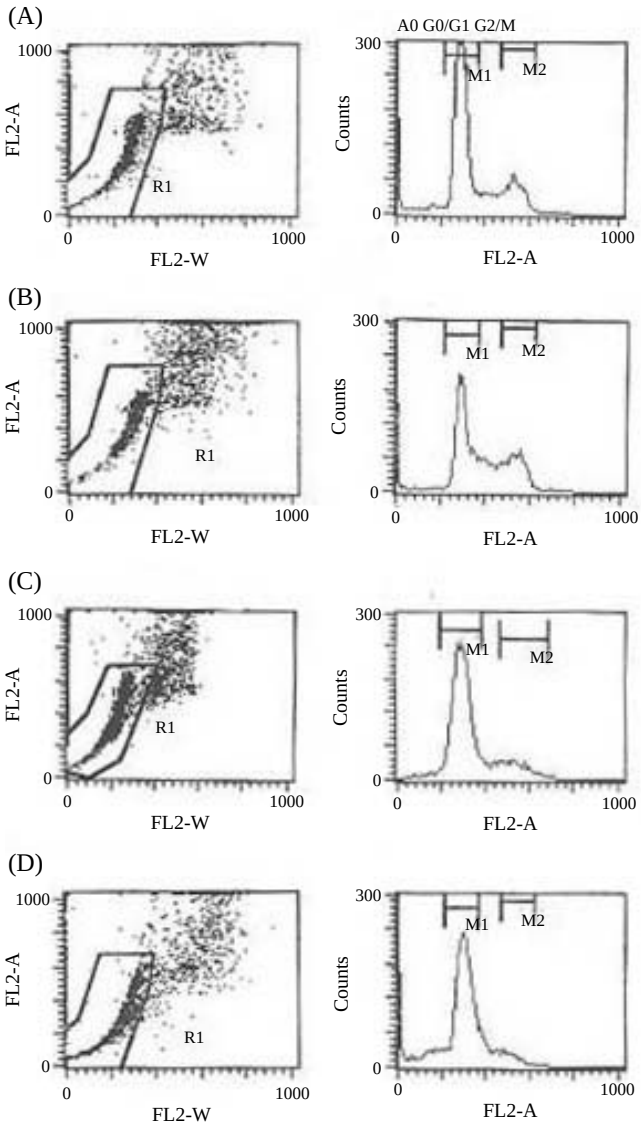

Fig. 4. Cell cycle analysis of HeLa cell follwing CMN and/or 5FU treatment for 24 hours. HeLa cells were treated with 0 (A), 5FU 1 μg/mL (B), CMN 1 μg/mL (C), 5FU 1 μg/mL+CMN 1 μg/mL (D) for 24 hours, stained with PI.

inducing tumor cell death via induction of apoptosis in sensitive cells (Sun et al., 1992; Chen et al., 1996; Zhu et al., 1997). Several studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents, including cisplatin, camptothecin, etoposide etc. There is accumulating evidence that the efficiency of anti-tumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis (Cohen, 1993). Although different chemotherapeutic agents are in clinical use, the situation demands a better drug, which can either independently or in combination with other drugs induce cell death or apoptosis selectively in cancer cells in an efficient manner.

CMN, a dietary supplement and food additive derived from the major yellow pigment of tumeric obtained from the curcuma longa linn plant and has been demonstrated to inhibit tumor initiation induced by various carcinogens. Previous studies have shown a dose-dependent inhibition of proliferation by CMN in Lovo cells, a colon cancer cell line (Chen et al., 1999; Jaiswal et al., 2002), breast cancer (Ramachandran & You, 1999; Choudhuri et al., 2002) and ovarian tumor cells (Zheng et al., 2002). Our study selected curcumin to study its role as an effective inducer of apoptosis and also to investigated the synergistic effects of CMN and chemotherapeutic agent 5FU on the growth suppression of human cervical cancer cells, HeLa. CMN induced cell death in HeLa cells and increases 5FU-induced apoptosis. The morphological hallmarks of apoptosis such as nuclear fragmentation and internucleosomal fragmentation of DNA were observed. Cervical cancer chemothery *in vivo* improved in cases with high p53 expression in the tumor tissue (Miyake et al., 2000). The restoration of p53 lev-

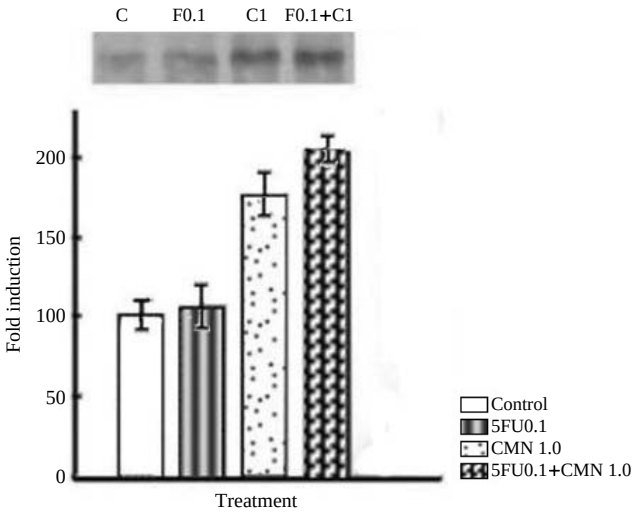

Fig. 5. P53 protein expression in HeLa cells treated with 5FU and/or CMN for 24 hours. p53 protein expression in HeLa cells after treatment with untreated control, 5FU 0.1 μg/mL, CMN 1 μg/mL, and 5FU 0.1 μg/mL+CMN 1 μg/mL for 24 hours by western blotting (upper). The histogram was measured by densitometry. The results are expressed as fold induction in 5FU and CMN treated cells compared to untreated cells. Bars indicate standard error. The results shown are mean±SD of three individual experiments.

els could be a potential strategy to increase chemoresponsiveness (Reddy et al., 2001). Few studies have shown an association between cervical cancer and p53 protein expression. Furthermore, the synergistic effects of 5FU and CMN on cervical cancer has yet to be explored. Our study proved interaction of apoptosis induction and expression level of p53 tumor suppressor gene in cervical cancer cells. It was observed that CMN 1 μg/mL doses induced increase of p53 in HeLa cells. There was G2/M arrest in response to chemotherapeutic drug 5FU and this arrest coincided with the increase in p53 expression. Reddy et al. (2001) reported there was complete G2/M arrest with cisplatin after 36 hours of vitamin C priming and also a significant increase in apoptosis indicating the restoration of pro-apoptotic function of p53. Another important finding of our study was that a combination of 10 μg/mL 5FU with 1 μg/mL CMN augments anticancer effects more efficiently than 5FU alone as evidenced by increased apoptosis and p53 expression in HeLa cells.

The present communication suggests a combination treatment synergism of great effectiveness by combining CMN and 5FU, this natural product may be an effective in the management of patients with advanced cervical cancer.

More study on the effect of this particular drug on cervical cancer should be conducted to provide additional information regarding its mechanism of action.

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＜국문초록＞

암은 전 세계적으로 사망률이 높은 질병으로 현재 자궁암 치료는 cisplatin, 5-fluorouracil (5FU) 등의 항암화학요법과 방사선 요법 등을 복합적으로 병행하고 있다. 최근 항암효과를 가지고 있는 자연식품의 섭취에 관심이 커지면서 커리의 주성분인 curcumin (CMN), 녹차의 주성분인 카테킨, 토마토의 주성분인 리코펜 등의 약리효과에 대한 연구가 활성화되고 있다. CMN은 동물실험 결과 항염증의 활성이 있고, 피부, 대장, 유방, 전립선 등에서 암으로의 진행을 억제한다는 보고가 있으나 인체 자궁암에서는 그 효과가 밝혀져 있지 않다. 본 연구는 항암제 5FU와 CMN이 인체 자궁암세포 HeLa에 미치는 영향을 apoptosis의 유도로 평가하고, p53유전자 발현율과의 상관관계로 확인하고자 시행하였다. CMN은 HeLa 세포의 성장을 억제하였으며, 5FU로 유도된 apoptosis의 발생률과 p53유전자의 발현률을 현저하게 증가시키는 것으로 나타났다 ($p < 0.05$). 이러한 연구결과는 CMN이 자궁암 치료제의 가능성이 있으며, 또한 5FU와 복합적으로 사용하면 항암제를 단독으로 투여하는 경우보다 자궁암 치료에 보다 효과적임을 강력히 시사하는 것이다.