



# Apoptotic Effects of Curcumin (Diferuloyl Methane) on Squamous Cell Carcinoma of the Cervix

Mohammad Shahid, Mohammed Naim, Mahboob Hasan, Najmul Islam\*,  
Tamkeen Rabbani\*\*, Aysha Mubeen

## Abstract

Apoptotic effects of curcumin (diferuloyl methane) on squamous cell carcinoma of the cervix. The present study was conducted in departments of Pathology and Biochemistry, JNMC, AMU, over a period of one and half yrs. Caspase-3 and TNF-alpha assay was performed on monocytes isolated from cervical carcinoma patients and cultured with curcumin; cytospins and sections from cervical carcinoma tissue cultured with curcumin were prepared for the morphological evidence of apoptosis. Curcumin in the doses of 500 microg/ml increased the caspase-3 levels and decreases the level of TNF-alpha in human cells. Cytospins and sections from cervical carcinoma tissue cultured with curcumin showed better differentiation and increased number of apoptotic cells as compared to non curcumin controls. Curcumin in a dose of 500 microg/ ml promoted apoptotic preparedness of human cells and induced apoptotic change in cervical carcinoma cells.

## Key Words

Curcumin, Cervical Carcinoma, Apoptosis, Caspase-3, TNF-alpha

## Introduction

Cervical cancer is one of the leading causes of cancer death in women (1). In the recent years many drugs are being studied for anti cancer apoptotic effects (2). Researches in the last decade have revealed that diferuloyl methane (curcumin) has some therapeutic potentials against the malignancies. Curcumin is reported to be suppressor of tumor angiogenesis (3), modulator of tumor necrosis factor (TNF) (4), and down regulator of antiapoptotic gene products (5). The present study aimed at evaluating apoptotic effect of curcumin on the squamous cell carcinoma of the cervix.

## Material & Methods

This study was conducted in the Departments of Pathology, Biochemistry, and Gynecology and Obstetrics, Jawaharlal Nehru Medical College, AMU Aligarh from January 2007 to August 2008. Total 62 cytologically diagnosed cases of carcinoma cervix were selected. Five ml blood sample in plane vial for monocyte caspase III

and TNF alpha estimations was collected at the time of cervical biopsy for final diagnosis in every case. Similar 5ml blood samples from 20 normal healthy adult female blood donors were used as controls for serum values. Peripheral blood mononuclear cells (PBMCs) from the control and test samples were isolated by density gradient sedimentation on Ficoll-Paque separation medium. On cyto staining, the PBMC were comprised of 90% non-adherent lymphocytes and 10% adherent monocytes. Thereafter, PBMCs (5x10<sup>6</sup> cells/well) were added in 12-well tissue culture plates (Costar Corp. Cambridge, MA) in complete RPMI-1640 medium, and were subsequently incubated at 37°C, 5% CO<sub>2</sub> for 1-2 hrs for adherence, washed to remove non-adherent cells and rested for overnight in RPMI-1640 medium having 2% autologous serum. Then, the adherent monocytes were co-cultured for 24 hrs with doses of 500µg/ml of curcumin and cultures devoid of curcumin served as control. Also, some cultures

From the Deptt. of Pathology, \*Biochemistry, \*\*Obstetric & Gynaecology, Jawaharlal Nehru Medical College, AMU, Aligarh UP  
Correspondence to : Dr. Mohammad Shahid, Senior Resident, Department of Pathology, JNMC, AMU, Aligarh UP India.



were pre-treated with Z-VADFMK (R & D Systems, Europe) which is a known inhibitor of caspase-3. Following 24 hrs, the above cells were lysed for 30 min at 40C in 0.5 ml of protein lyses buffer, which was prepared using MLB buffer (50 mM Mops, pH 7.0, 250 mM NaCl, 5.0 mM EDTA, 0.1% NP40, and 1.0 mM DTT) supplemented with protease inhibitors (1.0 mM PMSF, 5.0 micro g/ml leupeptin, 10 mM NaF, 5.0 mM Na-pyrophosphate, 1.0 mM Na-orthovanadate, and 20 mM -glycerophosphate). Thereafter, the suspension was centrifuged at 20,000 rpm for 15 minutes at 40C. Protein concentration was determined and the culture supernatants were stored at -200C until use.

Caspase-3 related protease activity in supernatant (monocyte cell lysates) was determined with the help of Caspase-3 cellular activity assay kit plus (Biomol, USA). The effect of Caspase-3 inhibitor (ZVAD- FMK) was also noted in the assay. The cleavage of the substrate was monitored spectrophotometrically at 405 nm and the activities were calculated according to the instruction of the manufacturers.

The amount of TNF- alpha in test and control supernatants was determined by use of a commercial ELISA Kit. This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-alpha was available pre-coated onto a microplate. Standards and samples were pipetted into the wells and any TNF-alpha present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF- alpha was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and the color developed in proportion to the amount of TNF-alpha bound in the initial step. The color development was stopped and the intensity of the color was measured. Cervical biopsy in each case was divided into two; one piece preserved in neutralized formalin for histopathological diagnosis and to serve as control. Another half of the biopsy was transferred vial containing 1ml of RPMI-1640 medium with 2 mM HEPES buffer and 2% autologous serum and 500 g/ml of curcumin added freshly. Test samples were incubated for 48 hours at 37°C. The test biopsy was then transferred to formaline vial, labeled as test and sent along with control to the Histopathology laboratory and processed for paraffin sections (3-5mm in thickness) by rotator microtome (Shandon Finesse) 315 and stained with Hematoxylin & Eosin.

## Results

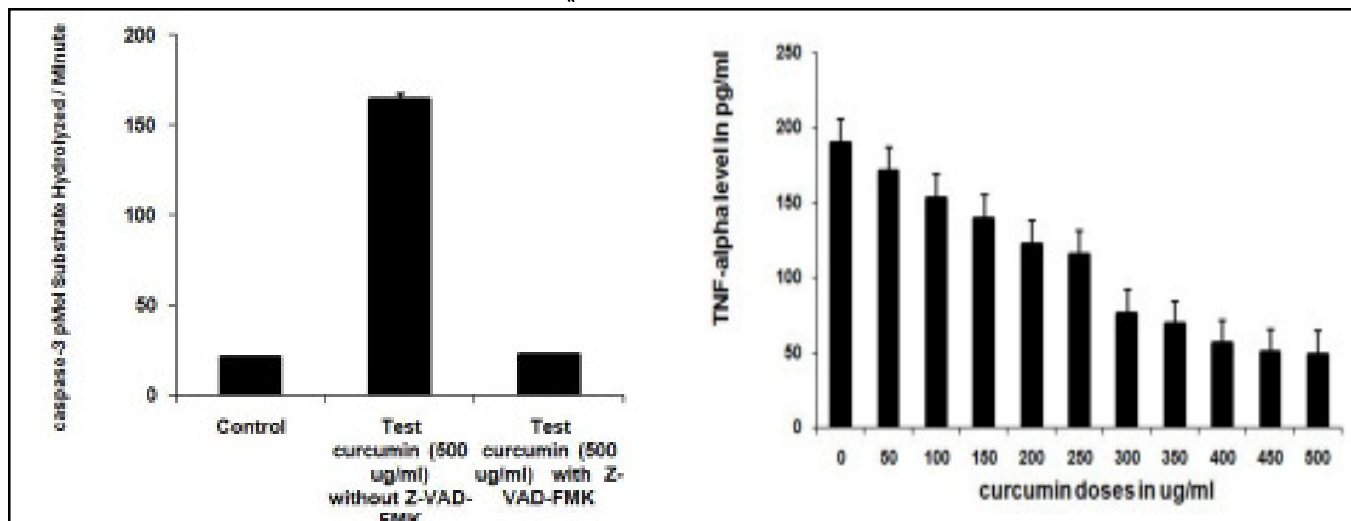
The activity of caspase-3 in curcumin treated and untreated monocytes extract samples from 62 patients of squamous cell carcinoma cervix is shown in the (Fig 1). Caspase-3 activity was significantly higher in the curcumin treated samples in comparison to not curcumin treated ones. Since caspase-3 is a known key enzyme involved in apoptosis, the rise of caspase-3 levels in curcumin treated samples implied that curcumin had apoptosis promotive effect in the bio-environment. Caspase-3 levels in cancer monocytes samples pre-treated with cell permeable inhibitor Z-VAD-FMK (caspase-3 inhibitor) followed by curcumin treatment for 24 hrs were markedly decreased (Fig 1). This finding was also affirmative of the caspase-3 promotive, hence, apoptotic effect of the curcumin.

An attempt was made to detect and quantify dose response effect of curcumin on the expression of soluble TNF-alpha protein in 24 hr culture supernatants of monocytes from cervical cancer patients (n=62) by ELISA, using curcumin in doses of 0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/ml respectively (Fig 2). The samples with 0µg/ml curcumin exhibited a high magnitude expression of TNF-alpha protein expression (190.22 pg/ml; p<0.001). On the contrary, varying doses of curcumin showed a linear suppression/inhibition of TNF-alpha protein expression. Co-culturing with 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/ml of curcumin showed TNF-alpha expressions of 171.22 pg/ml (p<0.001), 153.56 pg/ml (p<0.001), 139.67 pg/ml (p<0.001), 122.34 pg/ml (p<0.001), 115.45 pg/ml (p<0.001), 76.23 pg/ml (p<0.001), 56.23 pg/ml (p<0.001), 50.12 pg/ml (p<0.001) and 48.92 pg/ml (p<0.001) respectively. The IC<sub>50</sub> was found to be ~275 µg/ml.

Histomorphological effect of curcumin on cervical cancer cells in vitro, in RPMI-1640 medium for 48 hours, under light microscope, showed (Fig 3) rarely appreciable apoptotic cells in the control samples (n=62) with 38.8 ±1.53 apoptosis per 100 high power fields (hpf) in the range of 37-43/100 hpf. The test sections showed 40.58 ±0.31 apoptotic cells/100hpf in the range 37-45/100 hpf. The increase of apoptotic cells in test samples in comparison of the controls was significantly higher (p <0.0001).

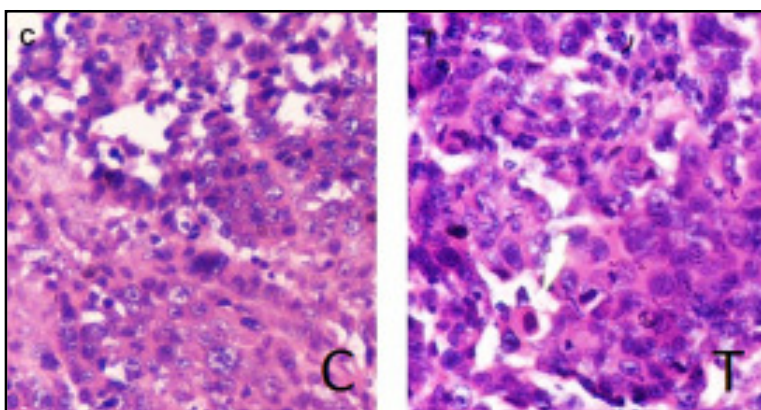
## Discussion

Apoptosis is one of the areas in carcinogenesis being investigated in anticipation of wide ranging implications and possible role in cancer prevention and therapy. Caspase-3 is recognized as one of the key molecules responsible for protein alterations and consequent



**Fig1. Bar Graph Showing Caspase-3 Activity in the Cancer Monocyte Lysate Supernatant in the Control Samples Without Curcumin, and 24 hrs Curcumin Treated Test Monocyte Samples Without and with Z-VAD-FMK Pretreatment**

**Fig 2. Showing TNF-alpha Expression in Lysate Supernatant of Cancer Monocytes Cultured for 24 hrs in the Control Samples Without (0 µg/ml) Curcumin & Test Samples Added Curcumin in the Doses 50 -500 µg/ml Respectively**



**Fig 3. Photomicrograph Showing Histomorphological Appearance of the Squamous Cell Carcinoma Cervix in the Control Without Curcumin, Showing Low Apoptotic (C) and Test (T) after 48 hrs Culture in the RPMI-1640 Medium with Curcumin Showing Prominent Apoptotic Cells (H&E X 400)**

apoptotic change in the cell. Apoptosis is characterized by cell shrinkage, membrane blabbing, chromosome condensation and nuclear fragmentation.

TNF- is a pro-inflammatory cytokine that has been implicated recently as both pro-apoptotic and anti-apoptotic factor. It is known that inflammatory process leads macrophages to secrete TNF-alpha , which induces apoptotic as well necrotic cell death in certain cancer cell lines. The TNF-alpha , thus, and caspase-3 enzyme levels may serve as indirect evidences of apoptotic change in a neoplasm (6).

Lin *et al* (2007) (7) studied effect of curcumin on orthotopic murine model of ovarian cancer and observed that it inhibits tumor growth and angiogenesis. In the

present study we observed that the monocytes from the patients of squamous cell carcinoma showed decreased viability in the culture medium on addition of curcumin. This suggested activation of the downstream signaling pathway of apoptotic monocyte cell death by the curcumin in the culture. Curcumin had been known to induce caspase activation, PARP cleavage and apoptosis in acute myeloblastic leukemia (8). In the present study recognition of PARP cleavage products by anti-PARP antibodies was inhibited by caspase inhibitors namely Z-VAD-FMK. This suggested that in cervical carcinoma under presented study the curcumin mediated apoptotic monocyte cell death was caspase dependent. The cleavage of PARP in response to activation of caspase-3, which was



curcumin stimulated, resulted in the loss of normal PARP function which irreversibly commits the cell to die (9, 10, 11). That the presently observed caspase-3 activation was due to curcumin induced PARP cleavage and not due to necrosis was confirmed by the results observed in cervical cancer monocytes pre-treated with pan-caspase inhibitor Z-VAD-FMK, where the inhibitor blocked PARP cleavage and in this case viability of monocytes was high (81.23%).

In many biological systems, caspase-3 acts as executioner caspase and cleaves intracellular proteins vital to cell survival and growth such as poly (adenosine diphosphate ribose)-polymerase (PARP) and inhibition of caspase like proteases prevent apoptosis. Present study demonstrated that curcumin activated the monocyte caspase-3 enzyme in the cervical carcinoma patients, indicating that both the intrinsic and extrinsic pathways of apoptosis were activated. This study also report suppression of high levels of TNF-alpha protein in cultures of cervical cancer monocytes by curcumin to be dose-dependent. In accordance with potential of TNF-alpha in cancer progression by rendering malignant cells to escape immune surveillance, and various types of cancers showing elevated levels, suppression of TNF-alpha by curcumin is affirmative of anti cancer effect of the curcumin. The anti-tumor immune response has been reported to be regulated by several factors, which includes cytokines produced by tumor and other cells of tumor stroma. It seems likely that the local cytokine microenvironment, acting on tumor cell or on the adjacent cells, can either block or facilitate tumor growth, and that proinflammatory cytokines strongly influence the immunologic state. Histopathological sections from Curcumin treated test samples showed evidences of apoptosis, showing one apoptotic cell per 2-3 high power fields to more than one dispersed apoptotic cells in a single high power field. Thus, the present study shows the chemo preventive apoptotic effect of turmeric polyphenol curcumin (diferuloyl methane) in cervical carcinoma cells. However, the exact mechanisms of the cancer preventive effects of curcumin are yet to be clearly understood and demonstrated, requiring further research.

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