EXPERIMENTAL
Materials:

N-acetyl-cysteine (NAC), H2O2, Agarose, Bovine serum albumin, Coomassie Brilliant Blue G-250 and R-250, p-nitrophenyl phosphate, anti-human IgG alkaline phosphatase conjugate, Tween-20, TEMED (N,N,N',N'-Tetramethylethylenediamine), dithiothreitol (DTT), Phenylmethylsulphonyl fluoride (PMSF), Sodium azide, Ethidium bromide, Cumene hydroperoxide were from Sigma Chemical Company, U.S.A. Caspase 3 and PARP were from Bioscience, USA. Caspase-9 was from Biovision, USA. Bcl-2, rabbit anti human antibody, was from Santa Cruz, USA. Fetal calf serum, antibacterial and anti fungal antibiotics were from Intergen, NY, U. S. A. Chloroform and Isoamyl alcohol were from Qualigens, India. Reduced glutathione, Glutathione reductase and reduced β-nicotinamide adenine dinucleotide phosphate were from HiMedia Chemical Company, India.

EGCG a Green tea polyphenol was obtained from Sigma Chemical Company, USA. RPMI-1640 medium were from HiMedia, India. MTT cell viability assay kit was from R & D Systems; U.S.A. 12-wells tissue culture plates were obtained from Techno Plastic Products (TPP), Switzerland. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were from NUNC, Denmark. All other chemicals were of the highest analytical grade available.

Study subjects:

Venous blood was obtained from healthy nonsmoking adult volunteers with no history of any disease. Also, blood from patients with cervical cancer was obtained from the patients admitted to J. N. Medical College Hospital of A.M.U., Aligarh. The diagnosis of cervical cancer was based on Pap smear and later confirmed by pathological examination of biopsies. Serum was separated and stored at -20°C until required.

Methods

1. Determination of protein concentration:

Protein was estimated by the methods of Lowry et al. (1951) and Bradford (1976).
This method is based on strong binding of the dye Coomassie Brilliant Blue G-250 in acidic medium, to protein hydrophobically, and at positively charged groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue color develops ($\lambda_{\text{max}}$-595 nm).

**Preparation of dye:**

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1.0 litre and filtered through Whatman No. 1 filter paper to remove undissolved particles.

**Procedure:**

To 1.0 ml of solution containing 10-100 ug protein was added 5.0 ml of dye solution. The contents were mixed thoroughly by vortexing. The absorbance was read at 595 nm after 5 minutes against a reagent blank.

**Polyacrylamide gel electrophoresis of proteins:**

Polyacrylamide gel electrophoresis was performed under denaturing conditions as described by Laemmli (1970).

**Reagents:**

(i) **Acrylamide-bisacrylamide (30:0.8)**

A stock solution of 30% acrylamide containing 0.8% bisacrylamide was prepared by dissolving 30 gm of acrylamide and 0.8 gm of bisacrylamide in a total volume of 100 ml. The solution was stored at 4°C in an amber coloured bottle.

(ii) **Resolving gel buffer**

A stock solution was prepared by dissolving 36.3 gm Tris base in 48 ml of 1N HCl. The contents were mixed, pH adjusted to 8.8 and the final volume brought to 100 ml with distilled water.
(A) Protein estimation by the Lowry (Folin-Ciocalteau) method:

Protein estimation by this method involves complexing of the protein's peptide bonds with Cu²⁺ under alkaline conditions (Lowry et al., 1951). The resultant Cu⁺ appears to catalyze the oxidation of tyrosine and tryptophan residues by reducing phosphomolybdotungstate anions in the Folin's reagent (a mixture of sodium tungstate, molybdate and phosphate), added subsequently. This reaction develops a blue color due to the formation of heteropolymolybdenum blue, which can be quantified by its absorbance at 660 nm.

Reagents:

(i) Folin-Ciocalteau reagent
The reagent was diluted 1:4 with distilled water before use.

(ii) Alkaline copper reagent
The components of alkaline copper reagent were prepared as follows:
(a) 2% sodium carbonate in 100 mM NaOH.
(b) 0.5% copper sulphate in 1% sodium potassium tartarate.

The working reagent was prepared fresh before use by mixing the two components in the ratio 50:1, respectively.

Procedure

To 1.0 ml of protein sample was added 5.0 ml of freshly prepared alkaline copper reagent. After thorough mixing, the reaction mixture was allowed to stand at room temperature for 10 minutes, followed by the addition of 1.0 ml of 1:4 times diluted Folin-Ciocalteau reagent. The contents were mixed immediately. The reaction was allowed to proceed for 30 minutes at room temperature and each tube was subsequently monitored at 660 nm. The protein content of the unknown sample was determined by using bovine serum albumin to construct a standard calibration curve.

(B) Protein estimation by the Bradford method:
(iii) Stacking gel buffer

6.05 gm Tris was dissolved in 40 ml distilled water, pH adjusted to 6.8 with 1 N HCl and the final volume adjusted to 100 ml with distilled water.

(iv) Electrode buffer

3.03 gm Tris, 14.4 gm glycine and 1.0 gm SDS were dissolved in distilled water, pH adjusted to 8.3 and the final volume made up to 1.0 litre with distilled water.

(v) Sample buffer

a. 6.0 gm of Tris was dissolved in 80 ml distilled water and pH adjusted to 6.8 with phosphoric acid. The final volume was brought to 100 ml with distilled water.

b. 1.0 mg of bromophenol blue and 12.5 ml of glycerol were added to 12.5 ml of the above solution. B-mercaptoethanol was added just before use.

Recipe for 12.5% Native Gel

<table>
<thead>
<tr>
<th>Reagents</th>
<th>12.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide (30:0.8)</td>
<td>4.1 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>150 μl</td>
</tr>
<tr>
<td>10% Ammonium persulphate (APS)</td>
<td>50 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>12 μl</td>
</tr>
</tbody>
</table>

The final volume was raised to 10 ml with distilled water.
Procedure:

The glass plates (18 cm x 16 cm) were soaked in chromic acid and thoroughly washed with tap water followed by a final rinse with distilled water and ethanol. The plates were dried and sealed with 1% agarose using 1.5 mm thick spacers. The reagents were mixed and poured between the glass plates, a well-forming comb was inserted immediately and the gel was left to polymerize at room temperature. After ensuring complete polymerization, the protein samples (20–100 µg) in one-fourth volume of sample buffer were electrophoresed at 80 volts at room temperature. The gels were stained using 0.25% Coomassie Brilliant Blue R-250 or with silver stain reagent.

Preparation of whole cell lysate:

Principle:

Keratins are insoluble proteins. Therefore they are obtained by solubilizing with detergents like SDS or urea. Therefore, a SDS-lysis buffer is used which has EDTA and EGTA, which chelates the calcium ions, and SDS denatures the proteins. The cell suspension was subsequently sonicated, to cause cell disruption by shear forces by high-pressure sound waves, shears the DNA that is then pelleted down by centrifugation. Therefore the supernatant will have only the proteins of the cells.

Materials:

- 1x PBS
- SDS lysis buffer
- 20mM Tris pH 7.2
- 5mM EDTA pH 8
- 5mM EGTA pH 8
- 0.4% SDS

Procedure:

1. The adherent cells in the dish are washed twice with 1X PBS.
2. 300 μL of SDS lysis buffer was added to the cells.
3. The adherent cells are scraped off with a rubber policeman.
4. The cell suspension was transferred into an eppendorf tube and incubated on ice for 45 min.
5. The cell suspension then centrifuged at 10,000 rpm for 10 min at 4°C.
6. The supernatant was taken in fresh tubes and was stored.

**Estimation of Proteins by Peterson Method**

**Principle:**

Peterson method is a modified Lowry's method. The principle behind this method is that the peptide nitrogen reacts with cupric ions under alkaline conditions and there is subsequent reduction of the Folin-Ciocalteau phosphomolybdic- phosphotungtic acid to heteromolybdenum blue by the copper catalysed oxidation of aromatic acids. Tartrate enhances the reduction of the Folin’s reagent by cuprous ions.

**Reagents:**

- Bovine Serum Albumin (BSA) (1mg/ml) stock solution.
- Folin-ciocalteau reagent (1:5 ratio)
- CTC solution:
  - CTC reagent : (20%Na-bicarbonate+0.2%copper sulphate+0.4% sodium-potassium tartrate)
  - 0.8N NaOH
  - 10%SDS
  - Distilled water
    (Each should be in ratio of 1:1:1:1)

**Procedure:**
1. Gradients of the standard BSA between 5μL and 25μL were taken and 10μL of samples were taken.

2. The volume of the samples and of the standard BSA was made up to 1ml with distilled water.

3. To all the tubes, 1ml of CTC solution was added.

4. The mixture was mixed well and was incubated for 10 min at room temperature.

5. 0.5mL of Folin - ciocalteau reagent was added to the mixture.

6. The mixture was mixed well and incubated for 30 min at room temperature in dark.

7. The absorbance was measured at 750nm.

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

**Principle:**

SDS-Page is the most widely used method for analyzing protein mixtures qualitatively. SDS is an anionic detergent. The mercaptoethanol in the sample buffer reduces any disulphide bridges present that are holding together the protein tertiary structure, and the SDS strongly binds to, and denatures, the protein. The original native charge is therefore lost completely. The sample buffer also contains an ionisable tracking dye, bromophenol blue (BPB), that allows the electrophoretic run to be monitored, and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the buffer to the bottom when injected into the loading well.

A stacking gel is poured on top of the resolving gel. The purpose of the stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. Once the proteins reach the separating gel as a result of the small pore size, the negatively charged protein-SDS complexes now continue to move towards the anode, and, because they have the same charge per unit length, they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the protein separate, owing to the molecular sieving properties of the gel.
The bromophenol blue dye is totally unretarded as it is a small molecule and therefore indicates the electrophoresis front.

After the electrophoretic run, the gel is stained with the Coomassie brilliant blue (CBB) staining solution, where the Coomassie dye binds non-specifically to the aromatic amino acids of the proteins. The acetic acid and methanol mixture fixes the proteins onto the gel so that they will not get washed whilst they are being stained. The excess stain is then eluted using the same acetic acid – methanol – water mixture in the absence of the dye.

Reagents:

- **30% Acryl amide- Bisacrylamide solution**
  - [Acryl amide – 29.2g
  - Bis-acrylamide – 0.8g]
- 1.5M Tris buffer- pH 8.8
- 1M Tris buffer – pH 6.8
- TEMED
- 10% Ammonium persulphate(APS)
- 10% SDS
- **Sample Buffer:**
  - 0.5M Tris pH 6.8 1.25mL
  - Distilled water 3.55mL
  - Glycerol 2.5mL
  - 10% SDS 2mL
  - Bromophenol blue(0.5% W/W) 0.2mL
- **5X Tank buffer:**
- Tris base (0.1M) 1.51g
- Glycine (1.25M) 9.4g
- 10% SDS 5mL
- The mixture was then made up to 100mL with distilled water.

**Staining Buffer:**

0.25% (w/v) CBB in methanol: water: acetic acid (45: 45:10 by volume) mixture.

- Molecular weight marker (Page ruler Unstained Protein Ladder - Fermentas-Cat No: SM0661)

**Resolving gel mix (for 10% gel):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide solution</td>
<td>3.33 mL</td>
</tr>
<tr>
<td>1.5 M tris pH8.8</td>
<td>0.9 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3.6 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>25 µL</td>
</tr>
<tr>
<td>10% APS</td>
<td>40 µL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

**Stacking gel mix:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide solution</td>
<td>750 µL</td>
</tr>
<tr>
<td>1M tris pH6.8</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>3mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µL</td>
</tr>
<tr>
<td>10% APS</td>
<td>20 µL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

**Procedure:**

1. The apparatus was fixed and checked for leakage
2. 5 mL of the resolving gel mix was poured which is followed by stacking gel
3. The comb was placed immediately.

4. When gel has polymerized, the comb was removed and the apparatus was set in the electrophoresis apparatus.

5. The tank was filled with the electrode buffer and the molecular weight marker (10μL) and the samples were loaded.

6. Electrophoresis was carried out at 200 V till the dye front reaches the end of the gel.

7. The gel was then removed and stained using Coomassie brilliant blue staining solution for 2 hours in a rocker.

8. The gel was then destained using the destaining solution.

**Western Blotting**

**Principle:**

Western blotting is an analytical method used to detect the specific protein present in the cell lysate. The proteins are resolved on a SDS-Polyacrylamide gel and are then electrotransferred on to the Polyvinylidene Fluoride (PVDF) membrane. PVDF membrane has non-specific affinity for amino-acids. Protein binding is based on the hydrophobic and charged interactions between the membrane and the proteins. After electroblotting, staining the membrane with the Ponceau staining solution checks the efficiency of transfer.

The membrane is then probed with the mouse anti-GFP antibody (primary antibody), after having it blocked with Bovine Serum Albumin (BSA), which binds specifically to the Green Fluorescent Proteins (if present on the membrane). The membrane is then probed with a secondary antibody (anti-mouse), which binds specifically to an epitope of the primary antibody. The secondary antibody is conjugated with the Horse-Radish Peroxidase (HRP) enzyme. Subsequently, on the addition of acridinium based ECL plus substrate, the enzyme catalyses the conversion of the chromogenic substrate into a product (acridinium esters), which produces luminescent signals. These signals are
captured in an X-ray film, which is then developed to visualize the signals. Bands will appear as dark regions on the developed film.

**Reagents:**

- **Transfer buffer**
  
  - Tris base (0.02M) 9.1 g
  - Glycine (0.2M) 43.2 g
  - Methanol 600 mL
  - 10% SDS 3 mL
  
  The volume is made up to 3L with distilled water.

- **Ponceau staining solution**
  
  - 0.1% Ponceau S in 1% acetic acid

- **10X Tris buffered saline (TBS):**
  
  - 1M Tris pH 8 50 mL
  - 5M sodium chloride 50 mL
  
  The volume was made up to 500 mL with distilled water.

- **TBST**
  
  - 0.1% Tween 20 in 1X TBS.

- **Blocking solution**
  
  - 3% BSA in 1X TBS

- **Antibody dilutions**
  
  The antibodies are diluted with appropriate amounts of 0.5% BSA in 1X TBS.

- **ECL plus Western Blot Detection System (Amersham, Cat. No: RPN2132).**

**Procedure:**

1. The protein samples were resolved using 10% SDS-PAGE and were
transferred to the gel transfer cassette.

2. PVDF membrane was pre-activated with 100% methanol.

3. The membrane was then washed with water and subsequently saturated with transfer buffer.

4. The membrane was placed on top of the gel in such a way that there is no air-bubble in between the gel and the membrane.

5. The cassette was fixed and was run at 100 V for 1 hour.

6. The membrane was washed with distilled water.

7. The membrane was then removed and stained with Ponceau staining solution.

8. The membrane was incubated with blocking solution for 1 hour in a rocker at room temperature.

9. The membrane was then incubated with the primary antibody (Mouse anti-GFP antibody) for 1 hour in a rocker at room temperature.

10. The membrane was then washed with TBST four times with an interval of 10 min each to remove the excess unbound antibodies.

11. The membrane was incubated with secondary antibody (anti-mouse antibody) for 1 hour at room temperature in a rocker.

12. The membrane was then washed with TBST four times with an interval of 10 min each to remove the excess unbound antibodies.

13. The membrane was given a final wash with 1X TBS for 10 min in a rocker at room temperature.

14. The blots were then exposed to Lumigen detection reagent - ECL plus reagent mixture (40:1 ratio) in dark and were incubated for 4 min at room temperature.

15. The signals were then captured in X-ray films and they were then developed.
Hela Cell Line:

HeLa cell lines of cervical cancer were brought from National Center for Cell Sciences, Pune, India. Cells were maintained in CO₂ incubator maintained at 5% CO₂ using RPMI 1640 Growth medium. Gentamycin and fetal calf serum (10%) were used in culturing. Cells were harvested after fifth day and aliquots of the stock were kept in liquid nitrogen until use. The viability of the stock remained >99% at 1 year. Before use, aliquots were defrosted, followed by equilibration at 37°C for 45 minutes.

Preparation of EGCG Solution:

Epigallocatechin 3-gallate (EGCG) was purchased from Sigma, USA. The EGCG was dissolved in distilled water, after that EGCG solution was passed through the membrane (0.22 µm) filtered for in vitro uses.

Preparation of RPMI-1640 medium:

Dehydrated RPMI-1640 medium of one unit vial (16.3 gm) (HiMedia, India), was suspended in 950 ml of tissue culture-grade water at room temperature with constant, gentle stirring until the medium was completely dissolved. The container was rinsed with tissue culture grade water to remove all traces of powder and added to the above solution. 3.7 gm sodium bicarbonate was added to the medium and stirred until dissolved. The final volume was brought to 1000 ml with tissue culture grade water. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 0.22 micron using positive pressure rather than vacuum to minimize the loss of carbon dioxide, and stored at 4°C until use.

Treatment with EGCG and viability assay:

The effect of EGCG (0–80 µg/ml) on the viability of HeLa cells was assessed by using MTT Cell Viability Assay Kit (R & D Systems) according to the manufacture’s instructions provided.
Reagents supplied in the kit:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT reagent</td>
<td>25 ml</td>
<td>2–8°C</td>
</tr>
<tr>
<td>Detergent reagent</td>
<td>250 ml</td>
<td>18–24°C</td>
</tr>
</tbody>
</table>

Assay procedure:

Adherent cells were gently scraped with RPMI-1640 medium. After this, 5 x 10³ cells/well were seeded in 96-well flat-bottomed plates and allowed to attach overnight. Cells were incubated in RPMI-1640 with 2% autologous serum containing EGCG (0, 20, 40 and 80 µg/ml) for 48 hours at 37°C, 10% CO₂. After 48 hours, 10 µl of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well and incubation was continued for an additional two hours. When a purple precipitate was clearly visible under the microscope, 100 µl of detergent reagent was added to all wells, including control wells and incubated for two hours in the dark at 20°C. After incubation, the precipitate was solubilized and the absorbance of the resulting solution was measured at 630 nm using a microplate reader. Control cells were treated exactly the same except that no EGCG was added to the wells. The percentage of viable cells was calculated by the formula as described by (Islam et al., 2000) and the results were expressed as “Viable cells (% of control cells)”.

\[
\text{Viable (\% of control cells) = } \frac{\text{Absorbance value of control cells}}{\text{Absorbance value of treated cells}} \times 100
\]
Trypan blue exclusion assay for Cell viability:

The exponentially growing Hela cells were seeded onto 12-well, flat-bottomed plates at a density of 5×10³/mL and allowed to attach overnight. The cells were treated with the EGCG of varying concentration (0-80 μg/ml) for 48hr at 37°C in a CO₂ chamber (5%). The cells were collected by trypsinization and counted by a hemocytometer (Shanghai, China) under light microscope using the Trypan blue dye exclusion method for viability. The cells taking up Trypan blue (dead cells), and cells excluding the dye (viable cells) were counted. Percentage of viable cells was calculated by the following formula:

\[
\text{% Cell viability} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained plus unstained)}} \times 100
\]

Growth Kinetic Assay:

Exponentially growing HeLa cells were seeded at 0.1 x 10⁶ cells/petri dish of 35 mm diameter for growth kinetics studies. Cells were treated with varying concentration of EGCG (0-80 μg/ml) at 37°C for continuous exposure for 24 and 48hrs. Total cell number of untreated cells at time of treatment was taken as initial value (N₀). Cells were harvested at every 24 hrs time interval up to 48 hrs by trypsinization and counted using hemocytometer. For determining the proliferation (Nₜ/N₀), where, N₀ is the total number of cells at treatment and Nₜ is the total number of cells at time t. Both floating and attached cells were counted and fixed in 80% chilled ethanol for cell cycle analysis.

Cell cycle analysis:

Progression of cells through different phases of cell cycle was also followed along with studies on cellular growth kinetics. Cells were washed with PBS by centrifugation at 600g for 5 min, fixed in 80% chilled ethanol and store at 4°C till measurement. Flow cytometric measurement of cellular DNA content were performed with ethanol fixed
cells using the intercalating DNA fluorochrome, propidium iodide (PI) as described earlier (Zolzer et al., 1995). Briefly, the cells (0.5-1 million) were washed with PBS after removing ethanol and treated with of RNase A (200μg/ml) for 30 min at 37°C. Subsequently cells were stained with PI (25μg/ml) for 15min at room temperature. Measurement were made with an argon laser-based Flowcytometer (FACS-Calibur Becton Dickinson San Jose, CA, USA) using the Argon laser (488nm) for excitation. Distribution of cells in different phases of cell cycle was calculated from the frequency distribution of DNA content by using the Mod fit Programme (Variety Software, CA, USA).

Annexin V binding:

Apoptotic cells were detected by the labeling of externalized phosphotidyl serine using annexin V-FITC in unfixed cells (Verma and Mazumder., 1995). Following treatments cells were harvested and aliquots of 10^5 cells resuspended in 100μl binding buffer (10mM HEPES/NaOH, PH 7.4, 10mM NaCl, 2.5mM CaCl2) and 5μl Annexin V-FITC and 10μl PI (50μg/ml) were added. After 15 min at room temperature 400μl of binding buffer were added to each sample and analyzed by flow cytometry. The percentage of Annexin V-positive and negative cells were estimated by applying appropriate gates and using regional statistics analysis facility provided in the cells Quest Software (Becton Dickenson, San Jose, CA, USA).

Preparation of PBMC and treatment of monocytes culture

Peripheral blood mononuclear cells (PBMCs) from blood of healthy donors and cervical cancer patients attending O. P. D. of J. N. Medical College and Hospitals, AMU, Aligarh were isolated by density gradient sedimentation on Ficoll-Paque separation medium as described by us earlier (Mesiter and Anderson, 1983; Hasan et al., 2006). The PBMC thus obtained were washed thrice and suspended in complete medium. By cytostaining, PBMC were comprised of 90% non-adherent lymphocytes and 10% adherent monocytes. Thereafter, PBMCs (5x10^6 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% CO2 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 varying doses of EGCG (0-25 μg/ml, dissolved in RPMI-1640 media). Cultures devoid of EGCG served as control. Also, some cultures were pre-treated with Z-VAD-FMK, Z-IETD-FMK and Z-LEHD-FMK, which are known inhibitors of caspase-3, caspase-8 and caspase-9 respectively. Following 24 hrs, the above cells were lysed for 30 min at 4°C in 0.5 ml of protein lysis buffer, which was prepared using MLB buffer (50 mM 3-(N-Morpholino)-propanesulfonic acid (MOPS), pH 7.0, 250 mM NaCl, 5.0 mM EDTA, 0.1% NP-40 (a detergent), and 1.0 mM DTT) supplemented with protease inhibitors (1.0 mM PMSF, 5.0 μg/ml leupeptin, 10 mM NaF, 5.0 mM Na-pyrophosphate, 1.0 mM Na-orthovanadate, and 20 mM β-glycerophosphate). Thereafter, the suspension was centrifuged at 20,000 rpm for 15 minutes at 4°C and supernatants were stored at −20°C. Protein concentration was determined and the culture supernatants were stored at −20°C until use.

Caspase activity assay:

Caspase-3, caspase-8 and caspase-9 related protease activity in cell lysates was determined as described elsewhere (Green and Reed, 1998; Ashkenazi and Dixit, 1998; Stennicke and Salvesen, 1998). The substrates employed were Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) (Biomol.), Ac-Ile-Glu-Thr-Asp-pNA (IETD-pNA) and Ac-Leu-Glu-His-Asp-pNA (LEHD-pNA) (Upstate Biotechnology, USA) for caspases-3, 8 and 9 respectively. Caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) were from R & D Systems Europe (Abingdon, UK). Briefly, 1x10^6 cells in 12-well plates were incubated with RPMI-1640 medium containing EGCG (5 μg/ml) for 24 h. Thereafter, cells were lyzed in 80 μl of ice-cold lysis buffer as described above. Twenty microliters of the supernatant was added to 100 μl assay buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol, pH 7.4. Ten microliters of respective caspase substrates were then added in the reaction system to final concentration of 0.2 mM. The cleavage of the substrate was monitored spectrophotometrically at 405nm and the activities were calculated according to the instruction of the manufacturer.
In order to determine the effects of specific caspase inhibitors on EGCG-induced apoptosis, Z-DEVD-FMK, Z-IETD-FMK and Z-LEHD-FMK were dissolved in DMSO as 20 mM stock solution and added to the culture medium to a final concentration of 50 µM as described earlier (Liu et al., 2002). After 2 hr pre-incubation, EGCG (5 µg/ml) was added and incubated for 24 h. The changes of EGCG-induced apoptosis in the presence of these inhibitors were determined as described.

Cytomorphological and histomorphological effects of EGCG on cervical cancer biopsies:

In the present study, biopsy pieces were taken directly from untreated cervical cancer patient (n=9) admitted for the first time and showing frank fungating growth. The Institutional Committee approved the study utilizing biopsies and blood collection. Biopsies from subjects showing differentiated keratinising and necrotising lesions were excluded from the study. Biopsy pieces were washed with buffered normal saline to remove debris, transferred to RPMI-1640 culture medium, bisected into 2 halves of 4-5 mm size to serve as test and control. The samples in 15-ml Falcon tissue culture tubes were subjected to treatment without and with EGCG (5 µg/ml) and cultured at 37°C. 5% CO₂ for 48 hrs. Thereafter, samples were processed for cytocentrifuged smear, and further processed for paraffin sections and staining.

Glutathione peroxidase (GPx) assay:

The activity of glutathione peroxidase (GPx) was measured as described elsewhere (Mohandas et al., 1984; Mates et al., 1999; Hasan et al., 2006). The oxidized glutathione (GSSG) produced during GPx reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was regarded as the rate of GSSG formation during the GPx reaction. Cancerous cell cultures as described above were co-cultured for 24 hours with or without 10 mM NAC and 0-80 µg/ml EGCG. Thereafter, cells were gently scraped with lysis buffer containing protease inhibitors (50 mM Tris/HCl, pH 7.4; 1 mM EDTA; 500 mM PMSF). The cell suspension was homogenized with a sonicator on ice and centrifuged at 10,000 rpm for 10 minutes. Protein concentrations of supernatants were determined by the method of Bradford with
BSA as the standard, and were subjected to GPx activity determination. The reaction mixture (1.0 ml) containing 50 mM potassium phosphate (pH 7.0), 1 mM sodium azide, 2 mM GSH, 0.2 mM NADPH, 1 unit/ml glutathione reductase, 1.5 mM cumene hydroperoxide, and 20–100 µl of samples were incubated at 25°C for 5 minutes. The reaction was initiated by the addition of cumene hydroperoxide. The kinetic change was spectrophotometrically recorded at 340 nm (37°C) for 3 minutes. GPx activity was calculated after subtraction of the blank value, as µmol of NADPH oxidized/minute/mg protein (U/mg protein).

Statistical analysis:

Results were analyzed by paired t-test and the data expressed as mean ± SEM of six experiments unless otherwise specified. P<0.05 was considered statistically significant.