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Emodin induces apoptosis of human cervical cancer cells through poly(ADP-ribose) polymerase cleavage and activation of caspase-9

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Abstract

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an active herbal component traditionally used in China for treating various ailments. Emodin exerts antiproliferative effects in many cancer cell lines and the actual molecular mechanism of which is still not clear. Since apoptosis could be a potential mechanism to explain these effects, we tested whether emodin induces cell death in human cervical cancer cells. Our results suggest that emodin exerts antiproliferative effects in human cervical cancer cells. Emodin inhibited DNA synthesis and induced apoptosis as demonstrated by increased nuclear condensation, annexin binding and DNA fragmentation in Bu 25TK cells in the presence of emodin. Moreover, we demonstrate for the first time in human cervical cancer cells that the apoptotic pathway involved in emodin-induced apoptosis is caspase-dependent and presumably through the mitochondrial pathway, as shown by the activation of caspases-3, -9 and cleavage of poly(ADP-ribose) polymerase.
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Keywords: Emodin; Apoptosis; Caspase; Cervical cancer; Bu 25TK

1. Introduction

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an active component isolated from the herb, *Polygonum cuspidatum*, traditionally used in China for the treatment of skin burns, gallstone, hepatitis, inflammation and osteomyelitis (Tsai, 2001; Yang et al., 1999). Emodin, is known to have antimicrobial, antiviral, anti-inflammatory, antioxidant, immunosuppressive, antiulcerogenic, antifungal and chemopreventive activities (Barnard et al., 1992; Lee, 2001; Malterud et al., 1993; Muller et al., 1996; Wang et al., 1998). Earlier workers demonstrated antiproliferative effects of emodin against many tumor cells (Chang et al., 1996; Huang et al., 1992; Kuo et al., 1997; Zhang et al., 1995). Relevant to its antiproliferative effects, emodin is also known to inhibit tyrosine phosphorylation of protein tyrosine kinases, p56lck and HER-2/Neu (Jayasuriya et al., 1992; Zhang et al., 1995). In addition, emodin inhibits the expression of c-*myc* mRNA in rat glomerular mesangial cells (Liu et al., 1996). Thus, emodin exerts antiproliferative effects in many cancer cell lines and, although different mechanisms have been proposed, the actual molecular mechanism is still not clear.

Apoptosis could be a potential general mechanism providing a mechanistic basis for the antiproliferative effects of emodin. Apoptosis or programmed cell death is characterized by cell shrinkage, chromatin condensation, DNA fragmentation and the activation of specific cysteine proteases known as caspases. Two pathways that converge on caspase-3, one involving caspase-8 and the other involving mitochondrial release of cytochrome *c* and activation of caspase-9 have been described (Sun et al., 1999). The aim of the study was to determine whether emodin could induce cell death exhibiting typical biochemical characteristics of apoptosis in human cervical cancer cells and to identify the pathway through which it is mediated. Our results indicate that emodin inhibits growth and induces apoptosis in human cervical cancer cells. Emodin induced nuclear condensation, annexin binding, DNA fragmentation, caspase activation and poly(ADP-ribose) polymerase cleavage, but did not activate caspase-8 in Bu 25TK cells.

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2. Materials and methods

2.1. Cell culture and maintenance

HeLa, Ca Ski, ME-180 and Bu 25TK were procured from the National Centre for Cell Science, Pune, India. The cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (Life Technologies, USA) containing 10% fetal bovine serum (Sigma, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C. For all experiments, Dulbecco's modified Eagle's Medium containing 5% fetal bovine serum was used.

2.2. Reagents and antibodies

Emodin was purchased from Alexis, San Diego, USA. Antibodies to caspase-8, and poly(ADP-ribose) polymerase were procured from Cell Signaling, USA. Spectrofluorimetric assay substrates for caspases-3, -8 and -9, AcDEVD-AFC, Z-IETD-AFC and AcLEHD-AFC, respectively, were bought from Calbiochem, USA.

2.3. Cell viability assay

Cell growth assays were carried out as described elsewhere with slight modifications (Anto et al., 2000). Cells grown in 96-well microtitre plates (5000 cells/well) were incubated for 48 h with or without different concentrations of emodin (25–100 μM). Then the medium was removed and fresh medium was added along with 20 μl of 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) (5 mg/ml) to each well. The plates were incubated for another 3 h and the formazan crystals formed were solubilized with MTT lysis buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide). The plates were placed protected from light, overnight at 37 °C in an incubator. The color developed was quantitated (Measuring wavelength: 570 nm, reference wavelength: 630 nm) with a 96-well plate reader (Bio Rad, USA). The cell viability was expressed as percentage over the control.

2.4. Thymidine incorporation assay

Cells were cultured and treated with emodin as above and [^{3}H]thymidine incorporation was measured as described (Anto et al., 2000). Briefly, [^{3}H]thymidine was added to each well (0.2 μCi/well) and incubated for 6 h. Thereafter, the culture medium was removed, washed twice with phosphate-buffered saline and the proteins were precipitated with 5% trichloroacetic acid. The supernatant was removed and after washing with ethanol, the cells were solubilized with 0.2 N NaOH and the radioactivity was measured using a β-scintillation counter.

2.5. Acridine orange/ethidium bromide and annexin/propidium iodide staining methods

Bu 25TK cells grown in 12-well plates (5×10^5 cells/well) were treated with 50 μM emodin for 24 h. After washing once with phosphate-buffered saline, the cells were stained with 100 μl of a mixture (1:1) of acridine orange-ethidium bromide (4 μg/ml) solutions. The cells were immediately washed once with phosphate-buffered saline and viewed under a Nikon inverted fluorescent microscope (TE-Eclipse 300).

For annexin/propidium iodide staining, the cells (10^4 cells/well) were seeded in 48-well plates and treated with or without emodin for 16 h. Then they were washed with phosphate-buffered saline and treated with 1 × assay buffer, annexin-fluorescein isothiocyanate and propidium iodide as per the protocol described in the annexin V apoptosis detection kit (sc-4252 AK) from Santa Cruz Biotechnology. After 10–20 min, washed with phosphate-buffered saline and the greenish apoptotic cells were viewed using a Nikon fluorescent microscope and photographed.

2.6. Single cell gel electrophoresis or comet assay

The comet assay was performed as described with minor modifications (Singh et al., 1988). Briefly, the cells (treated with or without emodin for 24 h) were pelleted and resuspended in 0.5% low melting point agarose at 37 °C and layered on a frosted microscope slide previously coated with a thin layer of 0.5% normal melting agarose and kept for 5 min at 4 °C. After solidification, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.5, 1% Triton X-100 and 10% dimethyl sulphoxide for 1 h at 4 °C). The slides were then electrophoresed for 20–30 min at 25 V. The slides were washed with 0.4 M Tris (pH 7.5) and stained with ethidium bromide (1 μg/ml) and observed under a Nikon fluorescent microscope.

2.7. Terminal deoxynucleotidyl transferase-mediated biotin dUTP nick end-labeling (TUNEL) assay for in situ DNA fragmentation

To detect apoptotic cells, in situ end-labeling of the 3′OH end of the DNA fragments generated by apoptosis-associated endonucleases was done using the Dead End apoptosis detection kit (Promega, Madison, USA). The cells were grown on cover slips and treated with or without emodin for 24 h. The cells were washed in phosphate-buffered saline and fixed by immersing slides in 4% paraformaldehyde for 25 min. They were then washed twice by immersing in fresh phosphate-buffered saline for 5 min. Cells were permeabilised with 0.2% Triton X-100 solution in phosphate-buffered saline for 5 min, washed twice in phosphate-buffered saline and then covered with 100 μl of equilibration buffer and kept for 5–10 min. The equilibrated areas were blotted

around with tissue paper and 100 μl of Terminal deoxynucleotidyl transferase (TdT) reaction mix was added to the sections on the slide and were then incubated at 37 °C for 60 min inside a humidified chamber for the end labeling reaction to occur. Termination of the reaction was done by immersing the slides in 2× SSC for 15 min. The slides were washed thrice, by immersing in fresh phosphate-buffered saline, for 5 min to remove unincorporated biotinylated nucleotides. The endogenous peroxidase activity was blocked by immersing the slides in 0.3% H_2O_2. After washing, horseradish-peroxidase-labeled streptavidin solution was added and the slides were incubated for 30 min. After incubation, the color was developed with the peroxidase substrate (H_2O_2) and the stable chromogen (diaminobenzidine). The slides were then mounted and examined with a light microscope.

2.8. Caspase-3, -8 and -9 assays

The activities of caspase-3, -8 and -9 were assayed as described earlier (Ito et al., 1999). Briefly, 10^6 cells treated with or without emodin were harvested, washed with phosphate-buffered saline and lysed in radioimmunoprecipitation buffer (10 mM phenyl methyl sulfonyl fluoride, 1 μg/ml of aprotinin, 100 mM EGTA, 100 mM sodium orthovanadate and 100 mM dithiothreitol). The supernatant was removed after centrifugation at 15,000 rpm for 10 min. Activities of caspases-9, -8 and -3 were detected by measuring the proteolytic cleavage of the fluorogenic substrates, AcLEHD-AFC, Z-IETD-AFC and AcDEVD-AFC, respectively. For this, 50 μg of each sample was incubated with caspase assay buffer and the appropriate substrate at 37 °C for 45 min. The caspase activity was measured using a

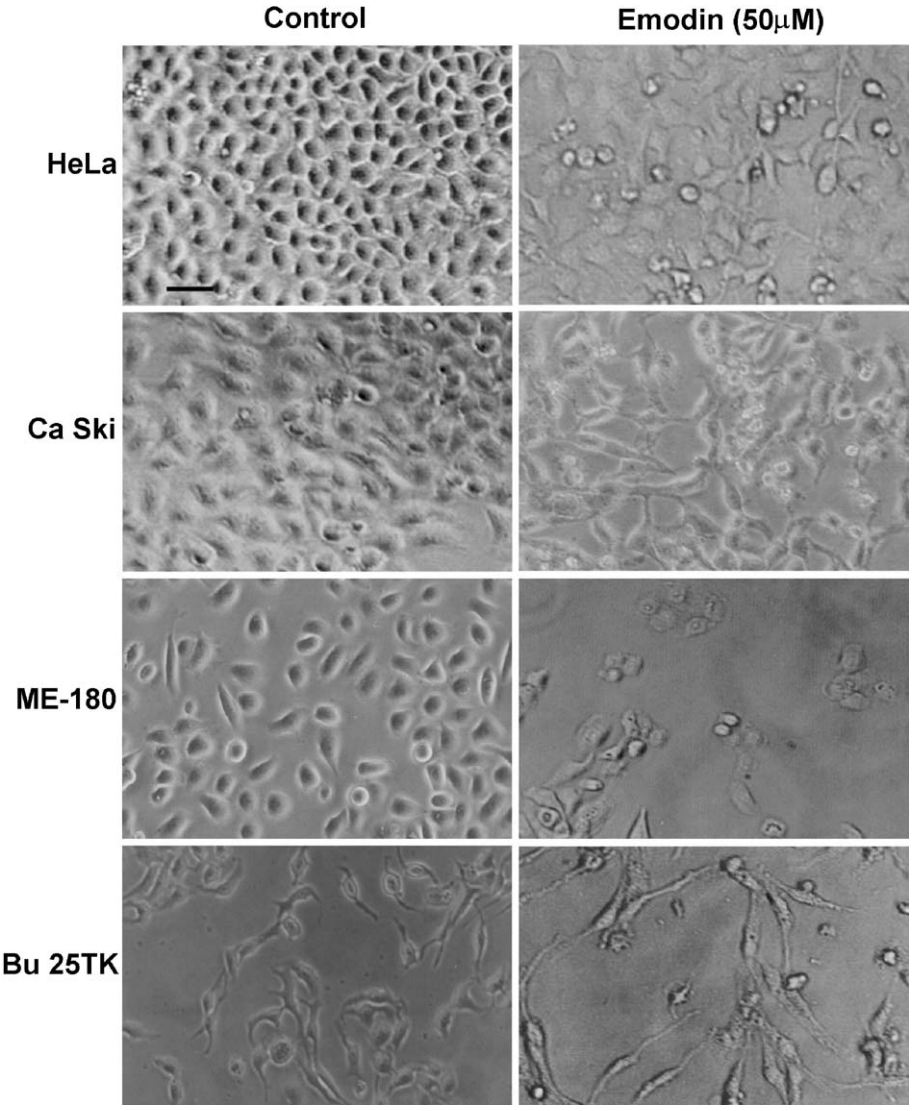

Fig. 1. Morphological changes induced by emodin in cervical cancer cells. Human cervical cancer cells, HeLa, Ca Ski, ME-180 and Bu 25TK, were seeded in 96-well plates. After 24 h, 100 μl of Dulbecco's modified Eagle's medium containing 5% fetal bovine serum was added without (control) or with 50 μM emodin. Cells were visualized in an inverted microscope after 48 h and photographed. The experiment was repeated three times with similar results. Bar = 50 μm.

spectrofluorimeter (LS-50B, Perkin Elmer, USA) set with an excitation at 400 nm and emission at 505 nm.

2.9. Western blot analysis

The cells treated with or without emodin were washed with phosphate-buffered saline and lysed in ice-cold radioimmunoprecipitation buffer. Whole cell extracts (60 μg protein) were resolved on sodium dodecyl sulphate-poly acrylamide gel electrophoresis, transferred to a nitrocellulose membrane and probed with anti poly(ADP-ribose) polymerase (1: 1000)/anti caspase-8 (1:3000) (Cell signaling, USA). Secondary antibody consisted of alkaline phosphatase conjugated secondary antibody (1:3000) (Sigma) and the bands were visualized as per the protocols given in the enhanced chemiluminescense detection kit (USB, Amersham, UK).

3. Results

3.1. Emodin induces cell death and morphological alterations in human cervical cancer cells

Human cervical cancer cells, HeLa, Ca Ski, ME-180 and Bu 25TK, were used in the present study. When 50 μM emodin treatment was given up to 48 h, many apoptotic bodies became visible and eventually some of the cells detached from the surface, but the untreated cells (control) were well spread with flattened morphology (Fig. 1). These results suggest that emodin-treated human cervical cancer cells undergo cell death and exhibit morphological features suggestive of apoptosis.

3.2. Emodin inhibits proliferation and DNA synthesis of cervical cancer cells in a concentration-dependent manner

To quantitate the effects of emodin on cell growth, cell viability was first assayed by reduction of MTT at 48 h after the addition of control medium or various concentrations of emodin. Cervical cancer cells exhibited a clear concentration-dependent growth inhibition with emodin, however with varying sensitivity (Fig. 2A). Ca Ski cells were least sensitive to emodin with an LD_{50} value of 98.4 μM followed by ME-180 (66.7 μM) and HeLa (61.2 μM), while Bu 25TK cells were highly sensitive with an LD_{50} value of 56.7 μM (Fig. 2A) and so this cell line was selected for all further experiments. Inhibition of cell proliferation may be mediated through inhibition of DNA synthesis and this was also studied by measuring the incorporation of [^{3}H]thymidine into DNA. As expected, DNA synthesis was inhibited by emodin in a concentration-dependent manner (Fig. 2B). These results indicate that emodin inhibits the proliferation and DNA synthesis of human cervical cancer cells in a concentration-dependent manner.

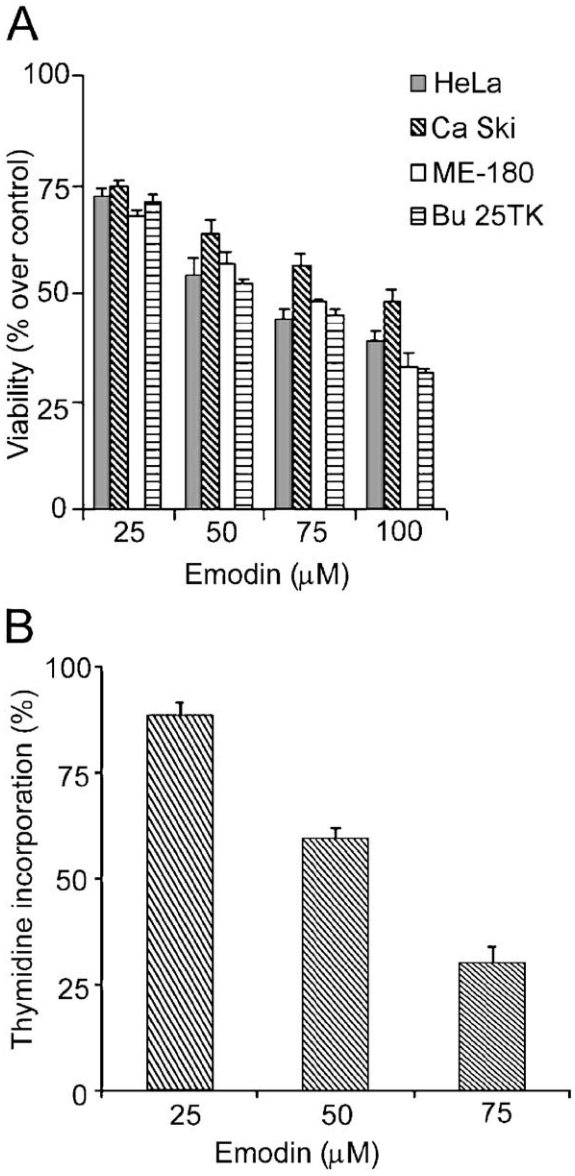

Fig. 2. Inhibition of cell viability and DNA synthesis by emodin. HeLa, Ca Ski, ME-180 and Bu 25TK cells grown in 96-well plates were treated without or with the indicated concentrations of emodin for 48 h. At the end of treatment, cell viability was assessed by MTT assay with quadruplicate samples as described in Section 2. The results are expressed as the mean percentage of control of quadruplicate determinations from three independent experiments and the error bars indicate standard deviation. The differences among the mean values were analyzed using one-way ANOVA followed by Tukey post-hoc analysis. The one-way ANOVA revealed that the average mean values of cell survival differed significantly as a function of concentration of emodin ($P < 0.0001$) (A). Bu 25TK cells were cultured and treated with or without emodin as above and incubated for 6 h with [^{3}H]thymidine. The proteins were precipitated with 5% trichloro acetic acid and, after washing with ethanol, the cells were solubilized with 0.2 N NaOH and the radioactivity was measured as described under Section 2. All results are expressed as the mean percentage of control ± S.D. of quadruplicate determinations from two independent experiments analyzed using one-way ANOVA followed by Tukey post-hoc analysis. The one-way ANOVA revealed that the average mean values of cell survival differed significantly as a function of concentration of emodin ($P < 0.0001$) (B).

3.3. Emodin induces nuclear condensation and translocates phosphatidyl serine to the outer membrane surface

To see whether the cytotoxic effects induced by emodin in Bu 25TK cells involve typical apoptotic changes, cells were first examined for changes in nuclear condensation. Upon treatment with 50 μM emodin, nuclear condensation (examined by staining the cells with ethidium bromide and acridine orange) was visible in Bu 25TK cells at 24 h, whereas the untreated control cells were healthy with very less staining (Fig. 3A).

We next looked for changes in phosphatidyl serine on the cell membrane by emodin since phosphatidyl serine flip flops on to the cell surface in the very early stages of apoptosis. Apoptotic cells were detected using annexin V labeled with fluorescein isothiocyanate since under defined salt and Ca^{2+} concentrations, annexin V can be used to bind phosphatidyl serine. Addition of propidium iodide helps to

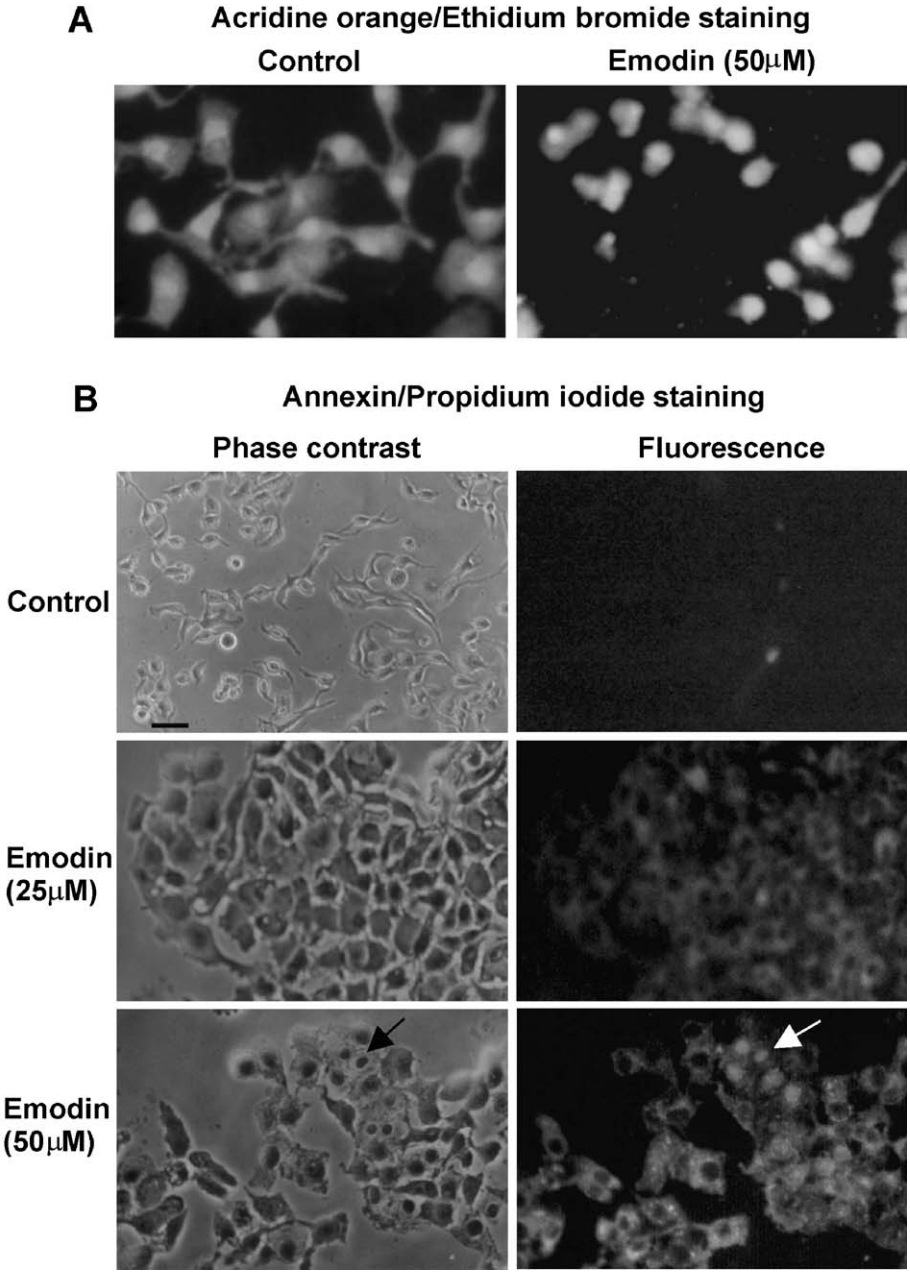

Fig. 3. Changes in nuclear morphology and annexin reactivity of Bu 25TK cells induced by emodin. Cells were seeded in 12-well plates and then treated with or without emodin for 24 h. After washing with phosphate-buffered saline, the cells were stained with a mixture of acridine orange-ethidium bromide mixture. The cells were viewed under an inverted fluorescent microscope and photographed as described under Section 2. The experiment was repeated two times with similar results (A). Bu 25TK cells were seeded in 48-well plates and treated with or without the indicated concentrations of emodin for 16 h. Then the cells were stained with annexin-fluorescein isothiocyanate/propidium iodide mixture using an apoptosis detection kit as per the manufacturer's protocol (Santa Cruz Biotechnology). These results were confirmed in another independent experiment (B). Bar = 50 μm.

distinguish the early apoptotic cells from late apoptotic or necrotic cells since propidium iodide cannot enter the cells in the early stages of apoptosis when the membrane integrity is intact. Bu 25TK cells treated with emodin (25 μM) clearly show early apoptotic changes like annexin binding (greenish yellow) on treatment with emodin while the control cells did not exhibit annexin binding (Fig. 3B). At 50 μM emodin, 28 ± 2.4% (mean ± S.D.) of cells showed propidium iodide staining (yellowish red) indicating the late stages of apoptosis. These results indicate that emodin induces nuclear condensation and translocation of phosphatidyl serine to the outer membrane surface in Bu 25TK cells.

3.4. Emodin induces DNA fragmentation in Bu 25TK cells

The degradation of DNA into multiple internucleosomal fragments of 180–200 base pairs is a distinct biochemical hallmark for apoptosis and to detect this, we used the single cell gel electrophoresis (Comet assay), a sensitive technique that allows detecting DNA strand breaks. DNA strand breaks create fragments, that when embedded in an agarose gel, migrate in an electric field. Cells with damaged DNA when stained with ethidium bromide appear like a comet and the length of the comet tail represents the extent of DNA damage. Emodin-treated cells showed well-formed comets while the untreated cells (control) did not demonstrate any comet like appearance (Fig. 4). To further confirm whether the DNA fragmentation is typical of apoptosis, we performed TUNEL assay that detects in situ DNA strand breaks induced by emodin treatment. The cells treated with emodin (50 μM) showed clear nuclear condensation, and also incorporated the labeled nucleotide into the DNA and the control cells without emodin did not show a positive TUNEL reaction (Fig. 4). The above results confirm that emodin induces DNA fragmentation, a typical feature of apoptosis in Bu 25TK cells.

3.5. Emodin induces poly(ADP-ribose) polymerase cleavage and activates caspases-3 and -9, but not -8

Caspases are a family of cysteine proteases that are activated during the execution phase of the apoptotic process. Once activated, caspases cleave and activate downstream caspases. We first looked for the activation of initiator caspases 8 and 9 by emodin in Bu 25TK cells. Caspase-9 activity was determined fluorimetrically and was significantly increased at 24 h of emodin treatment compared to the control (Fig. 5A). Caspase-3 was assayed using fluorogenic substrate, AcDEVD-AFC, an acetylated synthetic tetrapeptide corresponding to the upstream aminoacid sequence of the caspase 3 cleavage site in poly(ADP-ribose) polymerase and the flurophor AFC (7-amino-4-trifluoromethyl coumarin). Fig. 5B shows a concentration-dependent activation of caspase-3 in cells treated with emodin. However, caspase-8 was not activated by emodin treatment in Bu 25TK cells although curcumin used as positive control activated caspase-8 in these cells (Fig. 5C). We confirmed these results again by looking at the cleavage products of caspase 8 by Western blotting (Fig. 5D). We also examined the cleavage of a well-characterized caspase-3 substrate, poly(ADP-ribose) polymerase (116 kDa). It was processed to its predicted cleavage product of 89 kDa during emodin

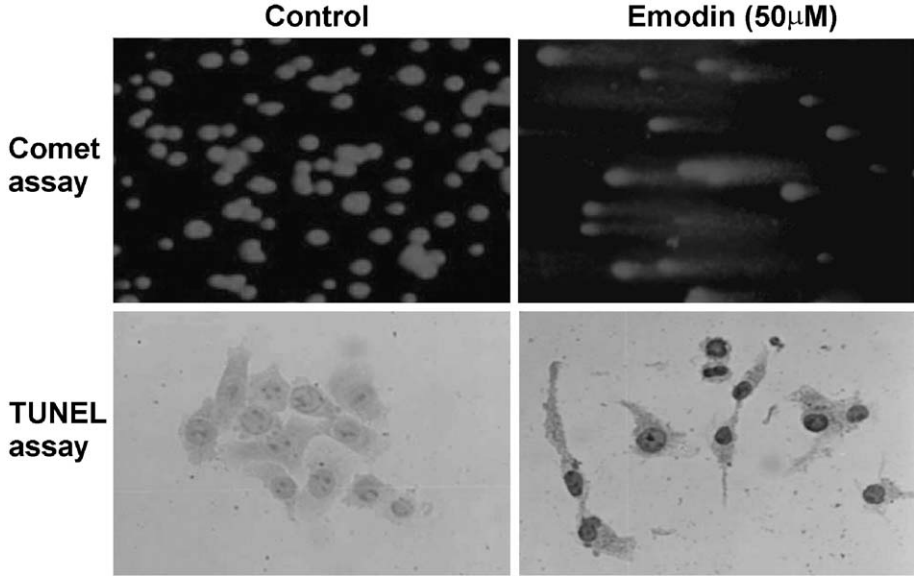

Fig. 4. DNA fragmentation induced by emodin as determined by comet and TUNEL assays. For comet assay, Bu 25TK cells (treated with or without emodin) were layered on a frosted microscope slide coated with agarose, immersed in lysing solution, electrophoresed, stained with ethidium bromide and observed under a Nikon fluorescent microscope as described under Section 2. For TUNEL assay, Bu 25TK cells were grown in cover slips and treated with or without emodin (50 μM) for 24 h. The cells were fixed, permeabilised with 0.2% Triton X-100, end-labelled with Terminal deoxynucleotidyl transferase reaction mix and the TUNEL reactivity was visualized as described in Section 2. Results are representative of three independent experiments.

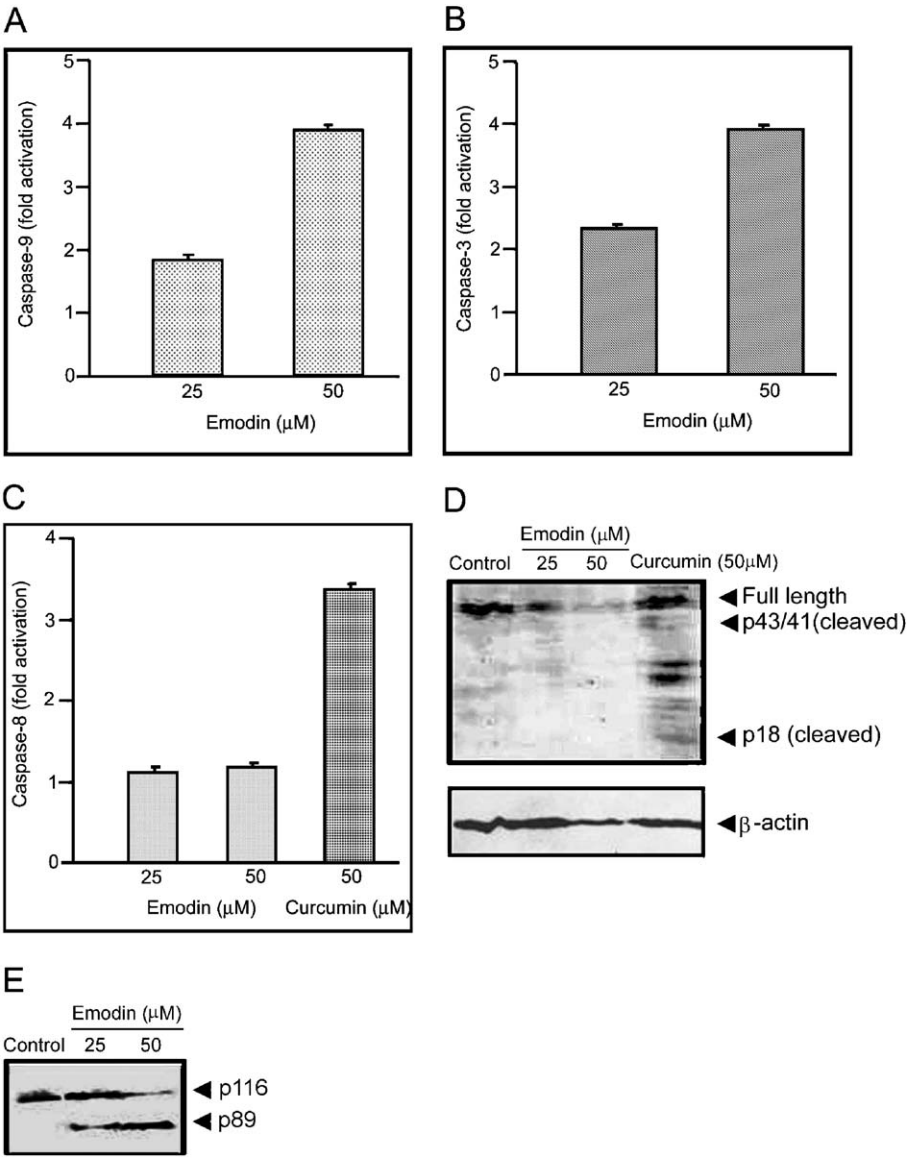

Fig. 5. Activation of caspases and cleavage of poly(ADP-ribose) polymerase by emodin. Bu 25TK cells treated for 24 h with or without the indicated concentrations of emodin were lysed and centrifuged and the supernatant was used for assaying caspase-9, caspase-3 and caspase-8 activities, shown in A, B and C, respectively, with appropriate fluorogenic substrates as described under Section 2. The experiment was repeated another time with similar results and the caspase activity was expressed as fold activation over the untreated control. The mean fold activation was significantly higher than the control sample ($P < 0.001$). Bars indicate standard errors. The cells were treated with or without emodin or curcumin (positive control) at the concentrations indicated, for 24 h, lysed, resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting was carried out to detect the cleaved products of activation of caspase-8, or poly(ADP-ribose) polymerase shown respectively, in D and E. The experiments were repeated at least two times with similar results and β-actin was used as loading control.

treatment, which was absent in the control cells (Fig. 5E). A band of 89 kDa was observed in Bu 25TK cells treated with emodin (25 μM or 50 μM) for 24 h (Fig. 5E). These results suggest the activation of caspases-3, and -9 but not -8 during emodin-induced apoptosis in Bu 25TK cells.

Taken together, these results suggest that emodin exerts antiproliferative effects in human cervical cancer cells and inhibits DNA synthesis in Bu 25TK cells. The cytotoxic effects of emodin are mediated through the induction of apoptosis as demonstrated by increased nuclear condensation, annexin binding and DNA fragmentation in Bu 25TK cells in the presence of emodin. Moreover, emodin-induced apoptosis is caspase-dependent presumably involving the mitochondrial pathway, as shown by the emodin-mediated activation of caspases-3, -9 and cleavage of poly(ADP-ribose) polymerase.

4. Discussion

There are not many studies focusing on the induction of apoptosis by emodin and the apoptosis-inducing effect of

emodin was first demonstrated in human kidney fibroblasts from lupus nephritis patients (Liu et al., 2000). A structurally similar compound, aloe-emodin, has also been shown to induce apoptosis in neuroectodermal cancer and lung carcinoma cells (Lee et al., 2001; Pecere et al., 2000). The present study demonstrated that emodin inhibits the growth of human cervical cancer cells and suggests that the anti proliferative effects of emodin are mediated through the induction of apoptosis. Moreover, we have shown for the first time in human cervical cancer cells, that emodin-induced apoptosis is caspase-dependent and presumably acts through the mitochondrial pathway. Consistent with our results, exposure of human kidney fibroblasts to emodin caused a concentration-dependent reduction in DNA synthesis (Ning et al., 2000). Recently, it has been demonstrated that emodin exerts potent cytotoxic effects on HL-60 cells mediated through the induction of apoptosis. Moreover, emodin induced rapid and transient induction of caspase-3 activity, but not caspase-1 and cleaved poly(ADP-ribose) polymerase (Chen et al., 2002). Similar to the present study, 50 μM emodin induced apoptosis in CH27, a human lung squamous carcinoma cell line, involving mitochondria and activation of caspase-3 and caspase-9 (Lee, 2001). However, in addition to caspase-9 activation, caspase-8 was also activated by emodin in CH27 cells, but not in Bu 25TK (human cervical cancer) cells (Lee, 2001). Further studies are needed to clarify whether emodin-mediated apoptotic response varies with cells of different tissue origin. Further studies are also in progress to understand the details of mitochondrial pathway stimulated by emodin in human cervical cancer cells. Although our data support the involvement of caspases, it remains to be tested whether emodin-induced apoptosis also operates through caspase-independent apoptogenic proteins like apoptosis inducing factor and endonuclease G (Du et al., 2000; Li et al., 2001; Susin et al., 1999). It will also be relevant to clarify the effects of emodin on cell cycle in human cervical cancer cells since emodin is known to block G1/S transition of cell cycle in human colon and kidney fibroblasts (Kamei et al., 1998; Ning et al., 2000) or G2/M transition of *ras*-transformed human bronchial epithelial cells (Chan et al., 1993). Interestingly, emodin enhanced the proliferation of MCF-7 cells (Matsuda et al., 2001), while another report suggested that it had no cytotoxic effect on MCF-7, HM02 (human melanoma) and HepG2 (human epidermoid carcinoma) cells (Demirezer et al., 2001). Emodin also enhanced sensitivity to chemotherapeutic agents in non-small cell lung carcinoma or breast cancer cells overexpressing *HER-2/neu* (Zhang et al., 1999). Further studies are still needed to understand the various mechanisms regulating the antiproliferative effects and apoptosis induced by emodin. Nevertheless, our experiments provide evidence that emodin is an effective inducer of apoptosis in human cervical cancer cells, Bu 25TK, through the activation of caspase-3 cascade, but independent of caspase-8 activation.

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