RESEARCH COMMUNICATION

Ethanolic Neem (Azadirachta indica) Leaf Extract Induces Apoptosis in the Hamster Buccal Pouch Carcinogenesis Model by Modulation of Bcl-2, Bim, Caspase 8 and Caspase 3

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Abstract

Induction of apoptosis is one of the most active strategies in cancer chemoprevention and the ability of medicinal plants in this regard has attracted major research interest. The present study was designed to investigate the apoptosis inducing capacity of an ethanolic neem leaf extract (ENLE) during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis using the apoptosis-associated proteins Bcl-2, Bim, caspase 8 and caspase 3 as markers. Topical application of DMBA to the hamster cheek pouch for 14 weeks resulted in well developed squamous cell carcinomas associated with increased expression of Bcl-2 and decreased expression of Bim, caspase 8 and caspase 3. Administration of ENLE inhibited DMBA-induced hamster buccal pouch (HBP) carcinogenesis, as revealed by the absence of neoplasms, with induction of Bim and caspases 8 and 3 and inhibition of Bcl-2 expression. Our results suggest that the chemopreventive effects of ENLE may be mediated by induction of apoptosis.

Key Words: Apoptosis - Bcl-2 - Bim - caspases - hamster buccal pouch carcinogenesis - neem extract

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Introduction

Cancer, the second most common disease that accounts for 7 million deaths per year worldwide, is associated with increased proliferation and decreased apoptosis (Vermeulen et al., 2003). Apoptosis is a complex process that involves many different signaling pathways and results in a multitude of changes in the dying cells. The apoptotic machinery is triggered as a result of a shift in the balance of anti- and pro-apoptotic proteins. Upregulation of antiapoptotic proteins, downregulation of proapoptotic proteins and decreased expression of caspases may lead to decreased apoptosis. Evasion of apoptosis is recognized to facilitate cancer development by blocking differentiation, promoting angiogenesis and increasing cell motility, invasion and metastasis (Ghobrial et al., 2005).

Induction of apoptosis is one of the active strategies to arrest proliferation of cancer cells. Radiation as well as agents such as tamoxifen that are capable of inducing apoptosis have been used to treat cancer (Woynarowska et al., 2000; Zhang et al., 2000). Many chemopreventive agents exert their anticarcinogenic effects by inducing apoptosis (Steele, 2003). Natural dietary constituents such as curcumin and resveratrol have been reported to induce apoptosis in malignant cells in vitro (Liontas and Yeger, 2004). In our laboratory, we have reported apoptosis induction by dietary agents in the hamster buccal pouch (HBP) carcinogenesis model (Balasenthil et al., 2002; Bhuvaneswari et al., 2004).

Of late, medicinal plants rich in antioxidant phytochemicals are being explored for apoptosis-inducing and cancer chemopreventive properties. *Azadirachta indica* A. Juss, commonly known as neem, widely distributed in Asia, Africa and other tropical parts of the world, has attained worldwide prominence owing to its medicinal properties. Neem elaborates a vast array of phytochemicals that are chemically diverse and structurally complex. The medicinal utilities have been described especially for neem leaf. Neem leaf has been reported to be non-toxic, non-mutagenic and found to possess immunomodulatory, anti-inflammatory and anticarcinogenic properties (Subapriya and Nagini, 2005). Previously, we documented the chemopreventive potential of aqueous neem leaf extract against experimental oral and gastric carcinogenesis (Balasenthil et al., 1999; Arivazhagan et al., 2001).

Several experimental studies have demonstrated that alcoholic extracts of neem leaf are more effective than

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aqueous extracts and exhibit a wide range of pharmacological properties (Chattopadhyay, 1998; Subapriya and Nagini, 2005). Recently, we demonstrated the protective effects of ethanolic neem leaf extract (ENLE) against MNNG-induced oxidative stress, genotoxicity and gastric carcinogenesis (Subapriya and Nagini, 2003; Subapriya et al., 2003, 2004b). We have also reported the chemopreventive potential of ENLE on HBP carcinogenesis (Subapriya et al., 2004a and 2005). The present study was designed to investigate whether ENLE induces apoptosis during 7,12-dimethylbenz[a]anthracene (DMBA)-induced HBP carcinogenesis using the expression of the apoptosis-associated proteins Bcl-2 and Bim as well as caspases 8 and 3 as markers.

Materials and Methods

Animals

The experiment was carried out with male Syrian hamsters aged 8-10 weeks weighing between 110-125g obtained from the Central Animal House, Annamalai University, India. The animals housed six to a polypropylene cage were provided food and water ad libitum and maintained under controlled conditions of temperature and humidity with an alternating light/dark cycle. All hamsters were fed standard pellet diet (Mysore Snack Feed, Mysore, India). They were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India and approved by the ethical committee, Annamalai University.

Chemicals

DMBA was purchased from Sigma Chemical Company, St. Louis, Mo, USA. All other reagents used were of analytical grade.

Collection of Plant Material

Fresh matured leaves of A.indica collected locally during March-April were identified by a pharmacognosy expert. These leaves were dried in the shade, powdered and the powders were used for the extraction. Voucher specimens were deposited at the herbarium of the Botany Department, Annamalai University.

Preparation of Neem Leaf Extract

The ethanolic extract of neem leaf was prepared according to the procedure described by Chattopadhyay (1998). Air-dried powder (1 kg) of A. indica leaves was mixed with 3L of 70% ethyl alcohol and kept at room temperature for 36 h. The slurry was stirred intermittently for 2h and left overnight. The mixture was then filtered and the filtrate was concentrated under reduced pressure (bath temperature 50˚C) and finally dried in a vacuum dessicator. The residue collected (yield 48g/kg of neem leaf powder) was a thick paste, green in colour and gummaceous in nature. The extract was suspended in normal saline to obtain a final concentration of 20 mg/mL and used for the experiment. The dose administered in the present study (200 mg/kg bw) is based on our previous work as well as reports in literature (Chattopadhyay, 1998; Subapriya and Nagini, 2003; Subapriya et al., 2003, 2004a;b,2005). This dose is also far less than the oral LD50 for ENLE, which was found to be 4.57g/kg bw in acute toxicity studies (Chattopadhyay, 1998).

Treatment Schedule

The animals were randomized into experimental and control groups and divided into four groups of six animals each. In groups 1 and 2, the right buccal pouches were painted three times per week with a 0.5 per cent solution of DMBA in liquid paraffin with a number 4 brush. Each application leaves 0.4 mg DMBA (Shklar and Oh, 2000). Group 1 received no other treatment. Group 2 animals received 200 mg/kg bw of ethanolic neem leaf extract by intragastric intubation three times a week on days alternate to the DMBA application from the beginning of the experimental period (Chattopadhyay, 1998). Group 3 animals received ENLE alone three times a week. Hamsters in group 4 painted with paraffin oil alone served as controls. The experiment was terminated at 14 weeks and all animals were killed by cervical dislocation after an overnight fast. Before an animal was killed, the right pouch was grossly inspected to evaluate premalignant lesions or tumour development and photographed. The buccal pouch tissues were subdivided and variously processed for distribution to each experiment.

Histopathology

Tissues were fixed in 10% formalin, embedded in paraffin and 2-3μm sections were cut on a rotary microtome and stained with hematoxylin and eosin. Basal cell hyperplasia, dysplasia and squamous cell carcinoma were diagnosed. Hyperplasia of oral epithelium was indicated by increased number of basal cells. Irregular epithelial stratification, increased number of mitotic figures, increased nuclear-to-cytoplasmic ratio and loss of polarity of basal cells characterized the dysplastic lesions. Squamous cell carcinoma (SCC) was diagnosed by the invasion of underlying tissues, nuclear pleomorphism and increased mitoses.

SDS-PAGE and Western Blot Analysis

Approximately, 50 mg of each tissue sample was subjected to lysis in a sample buffer containing 62.5 mM Tris (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and bromophenol blue. The protein concentrations of lysates were determined by Bradford (1976) method. SDS-PAGE was performed using equivalent protein extracts (55 μg) from each sample according to Laemmli (1970)]. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilion, Millipore, Bedfore, MA, USA). The membranes were incubated in TBS (150 mM NaCl/50mM Tris, pH7.4) containing 5% non-fat dry milk to block non-specific binding sites for 1 h. The blots were incubated with 1:1000 dilution

of anti Bcl-2, caspase 8 (Santa Cruz Biotechnology, CA, USA), caspase 3 (cell signaling, USA), and Bim (BD Biosciences CA, USA) overnight at room temperature. The blots were extensively washed with TBS containing 0.1% Tween-20. Bcl-2, Bim, caspase 8 and caspase 3 were detected by incubating with corresponding horseradish peroxidase-conjugated secondary antibodies (1:2000) for 30-45 min at room temperature. After extensive washes in TBS-T, the transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham, UK) according to the manufacturerûs instructions. Densitometry was performed on IISP flat bed scanner and quantitated with Total Lab 1.11 software.

Colorimetric Estimation of Caspase 3 Activity

DEVD-specific caspase 3 activity was assayed using CASP-3-C colorimetric kit (Sigma, St. Louis Mo, USA) according to the manufacturer's instructions. Cytosolic extracts were prepared by homogenizing tissues in lysis buffer containing 50mM HEPES (pH 7.4), 5mM CHAPS and 5mM DTT. The supernatant was collected as an enzyme source. The caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in release of the p-nitroaniline (pNA) moiety. The concentration of the pNA released from the substrate is calculated from the absorbance values at 405nm or from a calibration curve prepared with defined pNA solutions.

Statistical Analysis

The tumour burden was analysed using Student's t test. Statistical analysis for densitometric analysis was carried out using ANOVA followed by least significant difference test (LSD). The values are expressed as mean $\pm$SD. The results were considered statistically significant if the $p<0.05$.

Results

Table 1 shows the incidence of HBP tumours, mean tumour burden and histopathological changes observed in control and experimental animals. The incidence of HBP tumours in group 1 was 83.3 per cent (5/6 animals). All tumours were exophytic with a mean tumour burden of 213.16 mm^3. Although no tumours were observed in groups 2 and 3, two of six animals in group 2 had small multiple nodules.

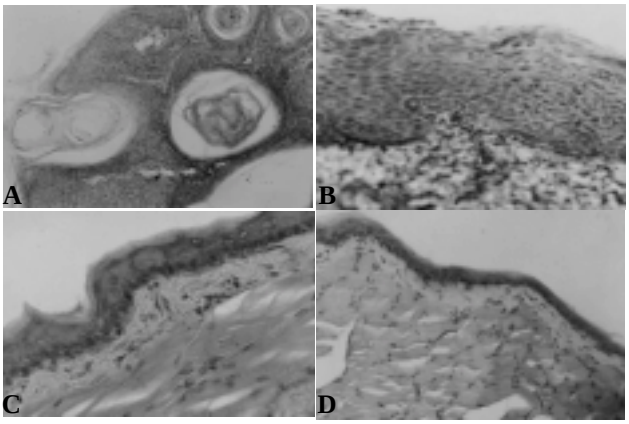


Figure 1. Microscopic Features of Buccal Pouch Mucosa of Control and Experimental Animals after 14 Weeks (H and E Stain X 20). (A) Photomicrograph of well-developed SCC exhibiting keratin pearls in the connective tissue of group 1 animal after 14 weeks of DMBA treatment (B) Photomicrograph of buccal pouch epithelium from a group 2 animal treated with DMBA and ENLE exhibiting moderate dysplasia (C) Photomicrograph of buccal pouch epithelium from a group 3 animal treated with ENLE alone exhibiting mild hyperplasia. (D) Photomicrograph showing normal buccal pouch histology of control animals (group 4)

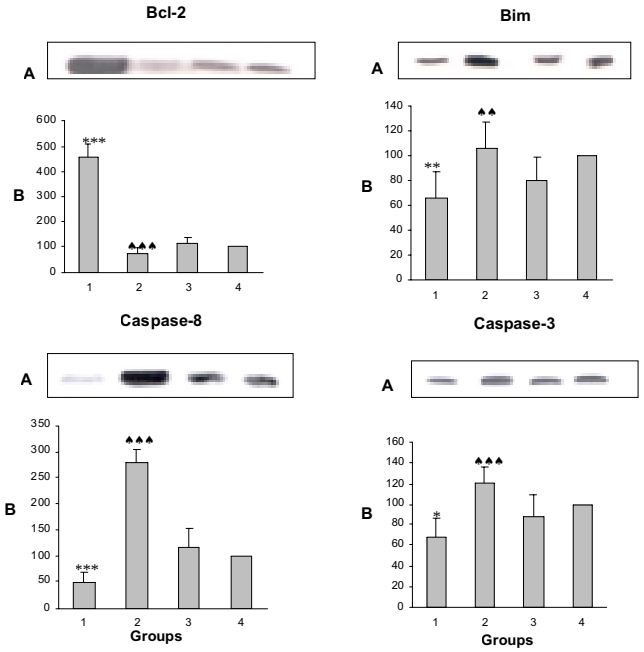
The tumours observed in group 1 animals were well-differentiated squamous cell carcinomas with evidence of keratin formation. Tumour cells consisting of pleomorphic, hyperchromatic nuclei exhibited altered nuclear/cytoplasmic ratio. Papillary projections of parakeratinised squamous epithelium into the connective tissue were also seen (Figure 1A). Although ethanolic neem leaf extract effectively suppressed development of HBP carcinomas, hyperplasia and dysplasia continued to persist in group 2 animals because of continual application of DMBA until the end of the experiment. However, these lesions were only mild to moderate (Figure 1B). In group 3, only 2 of six animals showed mild hyperplasia (Figure 1C) and the epithelium was normal in the remaining hamsters. In control animals, the epithelium was normal, intact and continuous (Figure 1D).

Figure 2 shows the effect of ethanolic neem leaf extract on Bcl-2, after Bim caspase 8 and caspase 3 expression in the buccal pouch mucosa of control and experimental animals. Using immunoblotting, expression of Bcl-2, Bim, caspase 8 and 3 was detected as bands with molecular weight

Table 1. Tumour Incidence, Mean Tumour Burden and Histopathological Changes in the Buccal Pouch of Hamsters in Experimental and Control Groups. (Mean $\pm$ SD; n = 6).

Group	Treatment	Tumour incidence	Tumour Burden[b] (mm^3)	Keratosis	Hyperplasia	Dysplasia	Squamous cell carcinoma
1.	DMBA	5/6 (83.3)	213.16 $\pm$ 24.21	+++	+++	+++	83.3%
2.	DMBA + ENLE	0	0	+++	++	+ to ++	-
3.	ENLE	0	0	-	+	-	-
4.	Control	0	0	-	-	-	-

b - Mean tumour burden was calculated by multiplying the mean tumour volume ($4/3\pi r^3$) with the mean number of tumors. (r= 1/2 tumour diameter in mm). ENLE = Ethanolic neem leaf extract.



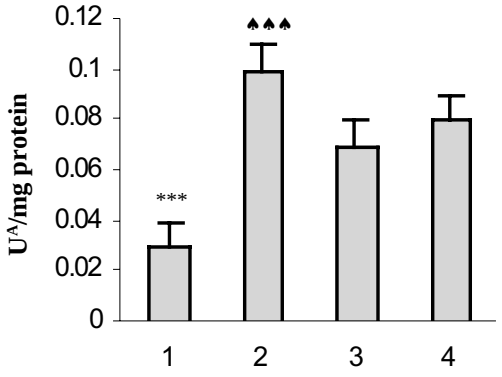
1) DMBA 2) DMBA + ENLE (200mg/kg bw)
3) ENLE alone (200mg/kg bw) 4) Control

Figure 2. The Effect of ENLE on The Expression of Bcl-2, Bim, Caspase-8 and Caspase-3 in the Buccal Pouch of Control and Experimental Animals (Mean ± SD; n=6).

(A) Representative immunoblots of Bcl-2, Bim, caspases 8 and 3. Protein samples (55µg/lane) resolved on 10% SDS-PAGE were probed with anti-Bcl-2, Bim, and caspases 8 and 3.

(B) Densitometric analysis. The mean protein expression from control lysates for six determinations was designated as 100% in the graph. Each bar for other experimental groups represents the mean protein expression ± SD of six determinations.

* Significantly different from group 4 ($p<0.05$)
** Significantly different from group 4 ($p<0.01$)
*** Significantly different from group 4 ($p<0.001$)
♠♠ Significantly different from group 1 ($p<0.01$)
♠♠♠ Significantly different from group 1 ($p<0.001$)



1) DMBA 2) DMBA + ENLE (200mg/kg bw)
3) ENLE alone (200mg/kg bw) 4) Control

Figure 3. The Effect of Ethanolic Neem Leaf Extract on the Activity of DEVD-specific Caspase-3 in the Buccal Pouch of Control and Experimental Animals (Mean ±SD; n=6)

*** Significantly different from group 4 ($p<0.001$)
♠♠♠ Significantly different from group 1 ($p<0.001$)
A µ moles of pNA formed/min

of approximately 25, 23, 57 and 32 kDa respectively. The mean protein expression from control lysates was designated as 100% in the graph (Figure 2B). Each bar represents the mean protein expression ±SD of six determinations per treatment performed after 14 weeks. Topical application of DMBA for 14 weeks significantly elevated Bcl-2 expression and decreased Bim, caspase 8 and caspase 3 expression in group 1 animals as compared with control animals (group 4). Administration of ENLE (group 2) significantly decreased Bcl-2 expression and significantly increased Bim, caspase 8 and caspase 3 expression compared to group 1. No significant changes in the expression of Bcl-2, Bim, caspase 8 and caspase 3 were observed in group 3 animals. Actin was used as a loading control. Representative immunoblots are shown in Figure 2A.

Figure 3 illustrates the activity of DEVD-specific caspase 3 in the buccal pouch in control and experimental hamsters. In DMBA painted animals (group 1), caspase 3 activity was significantly reduced as compared with control (group 4). Treatment with ENLE significantly increased caspase 3 activity in group 2 animals as compared with group 1. In animals administered ENLE alone (group 3), the activity of caspase 3 was not significantly different from that in control.

Discussion

In the present study, topical application of DMBA to the hamster cheek pouch for 14 weeks resulted in well developed squamous cell carcinomas with a very high tumour burden. This was associated with increased Bcl-2 expression and decreased expression of Bim, caspase 8 and caspase 3.

Bcl-2, a major anti-apoptotic protein inhibits apoptosis by preventing mitochondrial release of cytochrome C eventually resulting in inhibition of caspase activity (Kirkin et al., 2004). Bim, a BH3-only proapoptotic member of the Bcl-2 family induces apoptosis by binding to and inhibiting the function of antiapoptotic proteins such as Bcl-XL and Bcl-w. In addition, Bim has been reported to induce cytochrome C release from the mitochondria (O'Connor et al., 1998).

The release of cytochrome C from the mitochondria is also induced by caspase 8, an initiator caspase that links the death receptor and mitochondrial pathways of apoptosis. Caspase 3 is an effector caspase that executes cell death by cleavage of proteins vital for cell survival (Philchenkov, 2004). Overexpression of antiapoptotic proteins with downregulation of proapoptotic proteins and caspases has been documented in malignancies (Coultas and Strasser; 2003; Iolascon et al., 2003). Enhanced expression of Bcl-2 associated with diminished expression of Bim, caspase 8 and caspase 3 seen in the present study may facilitate evasion of apoptosis and development of the malignant phenotype in HBP carcinomas.

Administration of ENLE significantly reduced the incidence of DMBA-induced HBP carcinomas and tumour burden. ENLE downregulated Bcl-2 expression and upregulated Bim, caspase 8 and caspase 3 expression in the

buccal pouch indicating that it has apoptosis inducing effects in the target organ. The apoptosis inducing effect of ENLE may be attributed to upregulated immune surveillance and increased macrophage activity. Neem leaf extracts have been reported to enhance both humoral and cell mediated immune responses. In addition, extracts of neem leaf have been found to modulate both the classical and alternative C pathways and enhance the phagocytic activity of macrophages (Sen et al., 1992; Upadhyaya et al., 1993; Ray et al., 1996). The absence of apoptotic bodies in the DMBA + ENLE treated animals may be due to their engulfment by macrophages.

The results of the present study substantiate the anticarcinogenic effects of neem preparations reported by us as well as by others (Balasenthil et al., 1999; Tepsuwan et al., 2002; Subapriya and Nagini, 2003; Baral and Chattopadhyay, 2004; Dasgupta et al., 2004; Hanachi et al., 2004; Subapriya et al., 2004a; 2005). The chemopreventive effects of neem flowers on DMBA-induced mammary gland carcinogenesis and aflatoxin B-induced liver carcinogenesis have been documented (Tepsuwan et al., 2002). Dasgupta et al.. (2004) reported the chemopreventive potential of aqueous neem leaf extract in both benzo[a]pyrene-induced forestomach tumours and DMBA-induced skin papillomagenesis. Oral administration of 5 per cent neem extract was found to offer protection against diethylnitrosamine and acetylaminofluorene-induced hepatocellular carcinoma (Hanachi et al., 2004). Baral and Chattopadhyay (2004) reported a significant reduction in the growth of Ehrlich carcinoma and B16 melanoma cells by administration of neem leaf extract.

ENLE contains a number of antioxidants and anticarcinogens including terpenoids, limonoids, quercetin and sitosterols [9]. Constituents of neem leaf such as nimbolide, 28-deoxonimbolide and azadirone 1 have been found to possess cytotoxic activity against various cancer cell lines (Kigodi et al. 1989; Akudugu et al., 2001; Naundri et al., 2003). Quercetin, a highly ethanol soluble neem bioflavonoid and potent antioxidant has been reported to inhibit the growth of tumour cells in malignant cell lines and downregulate the expression of Bcl-2 and mutant p53 protein (Avila et al., 1994; Lamson and Brignall, 2000; Nguyen et al., 2004).

Our results provide evidence that ENLE induces apoptosis by a caspase-dependent pathway involving downregulation of Bcl-2 and upregulation of Bim, caspases 8 and 3 expression. A notable finding is lack of apoptosis induction by ENLE in the normal buccal pouch. In addition, there was no mortality or preneoplastic lesions in the animals administered ENLE alone. Mild hyperplasia in some of the ENLE alone treated hamsters may be due to trauma induced by storage of pellet diet in the cheek pouch, a habit often noticed even among control hamsters by us as well as by other workers (Butcher and Johnson, 1959; Kandarkar and Sirsat SM, 1990; Chandra Mohan and Nagini 2003). Furthermore, hyperplasia is generally not considered as a preneoplastic lesion unlike dysplasia (Pitot, 2002).

The results of the present study suggest that apoptosis induction may be a major mechanism through which neem leaf extract exerts its antiproliferative properties. However, additional studies on Fas and NF-κB signaling pathways and cell cycle-associated proteins are required to unravel the differential response of neem leaf extract in normal versus cancer cells to validate its chemopreventive potential.

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