Luteolin induces apoptosis in azoxymethane-induced colon carcinogenesis through the involvement of Bcl-2, Bax and Caspase-3

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ABSTRACT

Colorectal cancer (CRC) is a third most common of cancer in worldwide. A predictable mechanism for the development of CRC is an imbalance between cell renewal and cell death. Luteolin (LUT) is a bioflavonoid, has many beneficial effects such as antioxidant, anti-proliferative and anti-inflammatory. A novel approach is to develop a drug to induce apoptosis. In this present study is to evaluate the apoptotic inducing property of LUT in Azoxymethane (AOM)-induced CRC in Balb/c mice. The levels of lysosomal enzymes were analyzed. Induction with AOM increased the levels of lysosomal enzymes and subsequent administration with LUT decreased the levels of lysosomal enzymes. Expression of Bax, Bcl2 and caspase3 were analyzed. LUT administration increases the expressions of Bax and caspase 3 and decreased the expression of Bcl2. At this juncture, LUT act as a strong chemotherapeutic agent by modulating lysosomal enzymes and inducing apoptosis.

Key words: Apoptosis, Azoxymethane, Luteolin, Caspase 3

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men (663,000 cases, 10.0% of the total cancers) and the second in women (570,000 cases, 9.4% of the total cases) worldwide [1]. Limited data from the rural population based registries indicate that the incidence rate of CRC is very low in the rural settings. However the incidence rates of rectal cancer is disproportionately higher in rural India [2].

A predictable mechanism for the development of CRC is an imbalance between cell renewal and cell death, with proliferation being favored. A balance between new and old cells maintains organ size and colonic crypt structure [3]. Tumor growth depends not only on the rate of proliferation but also on the rate of apoptosis. For instance, Bcl-2 expression decreases upward along the crypts of normal colonic epithelium with the highest expression at the base and minimal amounts at the tip of the crypt [4]. This indicates the need to prevent programmed death to allow cell division at base of the crypts but stimulation of apoptosis as the cell matures and ages along the colonic crypt. Programmed cell death (PCD) is usually mediated through apoptosis, which is positively or negatively regulated by various extracellular factors. It has been demonstrated that a wide range of anti-cancer and chemopreventive agents induce apoptosis in malignant cells in vitro.

Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, and the activation of specific cysteine proteases known as caspases. Caspases, play a critical role during apoptosis. There are at least two major mechanisms by which a caspase cascade resulting in the activation of effector caspase-3 may be initiated by the most apical caspase, one involving caspase-8 and the other involving caspase-9 [5,6]. The Bcl-2 family consists of more than 30 proteins, which can be divided into three subgroups: Bcl-2-like survival factors, Bax-like death factors, and BH3-only death factors. Residues from BH1, 2, and 3 form a hydrophobic groove, with which BH3-only death factors interact through their BH3-domain, whereas the N-terminal BH4-domain stabilizes this pocket [7].
Cancer chemoprevention is a new promising strategy for cancer prevention by the use of either synthetic or naturally occurring chemicals to inhibit, reverse or retard tumor formation [8, 9]. There are a large number of phytochemicals present in our day today diets have shown antimutagenic and anticarcinogenic effects in numerous animal and cell culture systems [10-12]. However, epidemiological studies suggest that active agents found in the diet might reduce or increase the relative risk of cancer development [13].

Flavonoids are important phytochemicals characterized by a common benzo-γ-pyrene chemical structure and found widely in different plants, especially in genus Citrus [14, 15]. Luteolin, (3',4',5,7-tetrahydroxyflavone) is a common dietary flavonoid can be found in a variety of vegetables, fruits, and medicinal herbs [16](Sasaki et al., 2003). Luteolin has been shown to possess multiple biological activities such as anti-inflammation, anti-oxidant [17], anti-proliferative [18] and modulates the glycoproteins and status of thiols [19, 20] in AOM-induced colon carcinogenesis.

In this present study, we try to evaluate the apoptosis inducing property of LUT by assessing the expression of Bax, Bcl2, caspase3 and DNA fragmentation analysis also the status of lysosomal enzymes in AOM-induced CRC.

**EXPERIMENTAL SECTION**

**Chemicals**

Azoxyymethane was purchased from Sigma-Aldrich Chemical Company, St. Louis, USA. Luteolin was purchased from Cayman chemicals, USA. All other chemicals and reagents used were of analytical grade.

**Animals**

Male Balb/c mice weighing approximately 25-30 g were obtained from the Laboratory Animal Maintenance Unit, Tamilnadu Animal Science and Veterinary University, Madavaram, India. The animals were acclimatized to the laboratory conditions for a period of 2 weeks. They were maintained at an ambient temperature of 25±2° C and 12/12 h of light-dark cycle and were given a standard rat feed (Hindustan Lever Ltd., Bangalore) and water *ad libitum*. The experiments were conducted according to the ethical norms approved by Ministry of Social Justice and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (IAEC No. 01/021/08).

**Experimental procedure**

The animals were divided into four groups (n = 6 per group). Mice in group 1 served as control and received intra peritoneal injections (i.p.) of physiological saline. Group 2 mice were administered with AOM (15mg/kg body weight) intraperitoneally (i.p.) once in week for three weeks. Mice in group 3 (AOM + LUT) were treated with a single dose with 1.2 mg/kg body weight of LUT orally until end of the experiment, after AOM administration as mentioned in group 2. Mouse in-group 4 received the same dose of LUT as mentioned in group 3.

The experiment was terminated at the end of 17 weeks and all the animals were killed by cervical dislocation after an overnight fast. The tumorous colon tissue were excised out, the tissues were weighed and homogenized in Tris-HCl buffer pH 7.4 and centrifuged at 3000 rpm for 10 min. The supernatant obtained was used for various assays. A portion of the colon tissue was fixed in 10% neutral buffered formalin solution for histological studies.

**Assessment of lysosomal enzymes**

The tissue homogenate was subjected to lysosomes at 15,000 g for 10 min to collect the lysosomal fractions. The activities of lysosomal enzymes i.e. β-D-glucuronidase [21], β-D-galactosidase [22], β-D-N-acetylglucosaminidase [23], and acid phosphatase [24] were also assayed.

**Analysis of DNA fragmentation**

DNA fragmentation analysis was done by the method [25] previously described with little modifications.

**Immunohistochemical analysis of Bax, Bcl2 and caspase 3**

Paraffin embedded tissue sections of 4-micrometer thickness were rehydrated first in xylene and then in graded ethanol solutions. The slides were then blocked with 5% BSA in TBS (Tris buffered saline) for 2 h. The sections were then immunostained with respective primary antibody, diluted 1:500 with 5% BSA in TBS and incubated overnight at 4°C. After washing the slides thrice with TBS, the sections were then incubated with goat anti-rabbit secondary antibody in a dilution of 1:2000 with 5% BSA in TBS and incubated for 2 h at room temperature. Sections were then washed with TBS and incubated for 5-10 min in a solution of 0.02% diaminobenzidine (DAB) containing 0.01% hydrogen peroxide. Counter staining was performed using hematoxylin, and the slides were visualized under a light microscope (Nikon XDS-1B). To measure the relative intensity, scoring was done as
RESULTS

Luteolin modulates the levels of lysosomal enzymes

Figure 1 showed the levels of lysosomal enzymes in control and experimental animals. In AOM-induced (Group 2) mouse, there was a significant (p<0.001) increase in the activity of lysosomal enzymes such as β-glucuronidase, β-galactosidase, β-N-acetyl glucuronidase and acid phosphatase when compared to that of control (Group 1) mouse. Administration of LUT to the mouse significantly (p <0.001) decreased in the activities of lysosomal enzymes when compared to colon cancer (Group 2) bearing mouse. No significant changes were observed in control (Group 1) and LUT alone (Group 3) treated mouse.

![Figure 1. Effect of LUT on the levels of lysosomal enzymes in colon of control and experimental groups of animals](image)

Values are expressed as mean ± S.D. for 6 mice in each group. Activity is expressed as µmol of p-nitrophenol liberated/min/mg of protein for β-D-glucuronidase, β-D-galactosidase and β-D-N-acetylglucosaminidase. µmol of phenol released/h/100 of protein for acid phosphatase. *Control Vs AOM, †AOM Vs AOM+LUT; ns- non significant, p<0.05.

Luteolin induces DNA fragmentation

Genomic DNA of tissue homogenates of the experimental group of animals were ran on a 1.5% agarose gel electrophoresis was shown in Figure 2. Although not a distinct ladder, a moderate shearing was observed in the lane-3 of LUT-treated group of animals as compared to AOM-induced animals (lane-2). The results of this study suggest that LUT could induce apoptosis in colon cancer cells induced by AOM.

![Figure 2. Assessment DNA fragmentation of control and experimental groups](image)

Tissue sections were stained with the annexin-V-FITC-conjugated fluorescent dye (green). A tissue sections were also counterstained with PI (red) for nuclear staining. A) Control, B) AOM-induced showing decreased green fluorescence, C) AOM + LUT treated group shows increased green fluorescence, D) LUT treated group closely resembles as control.
Luteolin increases the expression of Bax and caspase-3 and decrease the expression of Bcl2

Figure 3A shows the immunohistochemical analysis of Bax, Bcl-2 and caspase-3 in control and experimental groups of animals. The expression of Bax and caspase-3 was negligible (Figure 3) in AOM-induced (Group 2) colon cancer bearing animals. In contrast the expression of Bcl-2 was increased notably. This indicates that, the decrease in the apoptosis in cancer status. Supplementation of LUT to cancer bearing animals (Group 3) showed an increased expression of Bax and caspase-3 was evident and the decreased expression of Bcl-2 was evident. No significant changes were observed in control (Group 1) and LUT alone (Group 4) treated animals. The immunohistochemical staining of Bax, Bcl-2 and caspase-3 were quantified and the result of the same is represented in figure 3B.

**DISCUSSION**

Lysosomes are cytoplasmic organelles containing acid hydrolases that are capable of degrading most cellular macromolecules to low molecular weight products. Lysosomes are reported to play an important role in cell death and tissue damage due to drugs [26-28] and toxins [29]. While lysosomes have been considered for many years as garbage compartments, it is now established that these organelles or their components can play an important role in numerous biological processes in eukaryotes such as programmed cell death [30] and secretion of cytotoxic molecules [31]. In our present investigation, AOM-induced (Group 2) mouse, there was a significant (p<0.001) increase in the activity of lysosomal enzymes such as β-glucuronidase, β-galactosidase, β-N-acetyl glucuronidase and acid phosphatase when compared to that of control was observed. But treatment with LUT reduced the levels of lysosomal enzymes.

The DNA ladder formation can be considered as the hallmark of apoptotic cell death. DNA fragmentation analysis illustrates increased DNA ladder formation upon agarose gel electrophoretic separation of DNA, indicating apoptotic cell death (Figure 2). Karrasch et al., [32] reported that LUT causes a DNA fragmentation in chemically induced colitis in rats. LUT showed morphologic alterations and DNA fragmentation [33]. An increase in the DNA fragmentation profile was observed in LUT treated mouse during study. This is clearly suggests that, LUT has the ability to induce apoptosis in vivo.
Controlled cell elimination or programmed cell death during development has been known for at least a century. This process was later recognized to be morphologically similar to cell death seen under some pathologic and toxicologic conditions, and it has been termed “apoptosis” to distinguish it from the common form of tissue injury, known as “necrosis” [34]. The process of apoptosis is a dynamic interplay of several molecules with upregulatory and downregulatory properties that is largely dependent on the cell type and the form of insult. No single factor in the machinery of apoptosis operates in isolation. It is unlikely that the activation or inactivation of a single component will alter the ultimate fate of the cell and lead to programmed cell death [35].

Previous observations demonstrated that a gradient increase of Bcl-2 as the tumor progressed in the adenoma-to-carcinoma sequence [36]. Subsequent studies showed that Bcl-2 expression increased only in the early stages of CRC progression and decreased as the tumor progressed into the late stages of the adenoma-to-carcinoma sequence [37, 38]. Bax, a pro-apoptotic member of the Bcl-2 family shares extensive amino acid homology with and acts as a functional antagonist to Bcl-2 [39]. Hence, the ratio of Bcl-2 /Bax appears to be the best variable in assessing the overall propensity of a cell to undergo apoptosis.

Caspase-3 is the most widely studied of effector caspases, it plays an important role in both death pathways and cleaves a wide range of cellular substrates, including structural proteins and DNA repair enzymes [40]. Caspase-3 is a critical component of the cell death machinery, being regarded as the most downstream enzyme in the apoptotic process due to its location in the protease cascade pathway [41]. The ratio of Bax/Bcl-2 is a critical determinant of the overall predisposition of a cell to undergo apoptosis. An increase in Bax relative to Bcl-2 promotes release of cytochrome C from the mitochondria with subsequent activation of caspase-3, thereby inducing mitochondrial mediated apoptosis [42-44]. Overexpression of Bax, and caspase-3 with downregulation of Bcl-2 by LUT showed the apoptosis-inducing potential of polyphenols. So natural compounds has the ability to induce cytotoxicity thereby protects against cancer [45, 46].

Sriram et al., [25] reported that, in vitro assessment of apoptosis, a garlic derivative diallyl sulfide mediates apoptosis via caspase-3 in colo DM 320 cell lines. Dietary administration of Scallion extract induces apoptosis [47] LUT enhances the caspase-3 in the colon of Dextran sodium Sulfide (DSS)-induced colitis [32]. In addition, LUT induces apoptosis in human myeloid leukemia cells [48], where it was observed by the detection of morphologic alterations and DNA fragmentation, although it was not elucidated how LUT caused this effect. Thus, LUT is an important cancer chemopreventive agent that induces apoptosis in tumor cells, but little is known about the molecular and biochemical mechanisms responsible for this activity [33].

**CONCLUSION**

In order to elucidate the mechanism by which LUT induce apoptosis, proteins were isolated from the colon were analyzed by immunohistochemistry and western blot. The results from this experiment revealed that LUT was able to increase active caspase-3 and Bax expression along with a concomitant decrease in Bcl-2 proteins levels, strongly suggesting that this compound induces apoptosis via the mitochondrial pathway.

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