RESEARCH COMMUNICATION

Effect of Luteolin on the Levels of Glycoproteins During Azoxymethane-induced Colon Carcinogenesis in Mice

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Abstract

Luteolin (LUT), a bioflavonoid has been used as a chemopreventive agent world-wide against chemically induced cancer. Hence we designed an experiment to assess chemopreventive action of LUT on lipid peroxidation (LPO) and glycoconjugates in azoxymethane (AOM)-induced colon carcinogenesis. Colon cancer was induced by 15 mg/body kg. body weight of AOM and administration of LUT (at the dose of 1.2 mg/kg. body weight) was till end of the study. Analysis of lipid peroxidative end products such as protein carbonyl (PC), malonadehyde (MDA) and conjucated dienes (CD) demonstrated significant increase in in AOM-induced animals with reduction by LUT ($p<0.05$) . Increased levels of glycoconjugates such as hexose, hexosamine, sialic acid, fucose and mucoprotein were analyzed in serum and colon tissues examined histopathologically by periodic acid Schiff's (PAS) staining were also reversed by LUT l($p<0.05$) . The secondary marker of colon cancer mucin depleted foci (MDF) was assessed in control and experimental group of animals. A characteristic increase of MDF was observed in AOM-induced colon cancer animals. Treatment with LUT decreased the incidence of MDF. These results suggest that LUT alters the expression of glycoconjugates and suppress colon cancer. Hence, we speculate that LUT can be used as a chemopreventive agent to treat colon cancer.

Keywords: Glycoconjugates - luteolin - azoxymethane - colon cancer - lipid peroxidation

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Introduction

Cancers of the large and small intestine are major contributors to worldwide cancer morbidity and mortality (Greenlee et al., 2000). Despite the development of new screening strategies, aggressive surgical and adjuvant therapy, and intensive research effects, little progress has been made in the successful management of this disease (Williams et al., 1999). The cure rate for this cancer has remained at 50% for some decades (Burnstein, 1993). The etiology of colorectal cancer is complex and may be attributable to combined actions of inherited and environmental factors. Epidemiological studies have indicated that colorectal cancer is strongly associated with diet (Slattery et al., 1999), and thus it may be possible to prevent the occurrence of this cancer by dietary modifications (Tanwar et al., 2009). Chemoprevention refers to the use of natural or synthetic compounds to prevent, reverse, or delay the development of cancer (Swan & Ford, 1997). Because food derived products exist universally and are expected to be safe, they are highly interesting for development as chemopreventive agents to treat cancer (Sengupta et al., 2002; Prabhu et al., 2009; Chihara et al., 2010).

Luteolin (3', 4', 5, 7-tetrahydroxyflavone), an important member of the flavonoid family, is present in various fruits and vegetables and has contributed to the antioxidant activity of artichoke leaf extract on reactive oxygen species in human leucocytes (Perez-Garcia et al., 2000). Luteolin is also reported to have anti-inflammatory properties and mediates its action by inhibiting of nitric oxide production (Kim et al., 1999). Luteolin has anti-allergic properties (Kimata et al., 2000a), and luteolin acts as a potent inhibitor of human mast cell activation through the inhibition of protein kinase C activation and Ca^{2+} influx (Kimata et al., 2000b). It is also reported to have anti-inflammatory/anti-allergic (Veda et al., 2002), anti-tumorigenic (Yasukawa et al., 1989) and antioxidant properties (Shimoi et al., 1994).

The importance of glycoproteins in bringing about neoplastic transformation is well documented. Glycoconjugates are necessary for the assembly of the oligosaccharide moieties of the glycoprotein chains and their levels have been found to be elevated in neoplastic conditions and can therefore be designated as non-specific markers of malignancy (Sen et al., 1983). Its levels are high in tumor tissue due to increased lipid peroxidation resulting in lowered antioxidant status (Scholz et al., 1979) and aberrant glycosylation (Hakomori, 1996). Carbohydrates moieties of glycoproteins have also been implicated in the transport of metabolites across cell membranes and also observed a direct relationship

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between glycoproteins and tumorigenesis (Femia et al., 2004). Previous studies from our laboratory, showed LUT decreases the incidence of aberrant crypt foci (Ashokkumar & Sudhandiran, 2008), reduces the tumor formation and inhibits the cell proliferation via Wnt/β-catenin mediated pathway (Ashokkumar & Sudhandiran, 2011) In the present study, evaluates the modulating effect of LUT on MDF, glycoconjugates and lipid peroxidation in AOM-induced colon cancer.

Materials and Methods

Chemicals

Azoxymethane 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 obtained from the Laboratory Animal Maintenance Unit, Tamilnadu Animal Science and Veterinary University, Madavaram, India and used for this study. 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 hours of light–dark cycle and given standard feed (Hindustan Lever Ltd., Bangalore) and water *ad libitum*. The experiments were conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (IAEC No. 01/021/08).

Experimental procedure

All mouse were divided into four groups (n = 6 per group). Group I - served as control animals and received intra peritoneal injections (*i.p.*) of physiological saline. Group II - (AOM) mouse were administered AOM (15mg/kg body weight) intraperitoneally (*i.p.*) once in week for three weeks. Group III - (AOM + LUT) were treated with a single dose of LUT (1.2 mg/kg body weight) orally until end of the experiment, after AOM administration as mentioned in group II. Group IV received the same dose of LUT alone as mentioned in group III.

A portion of the colon tissue was fixed in 10% buffered neutral formalin solution for histological studies. Blood was collected and serum was separated for further analysis of glycoconjugates.

Biochemical analysis

The protein carbonyl was estimated by the method of Levine et al. (1995), conjugated dienes by Kelin (1970) and malonaldehyde was estimated by the method of Okhawa et al. (1979). Hexose was estimated by the method of Niebes (1972). Hexosamine was estimated by the method of Wagner (1979). Sialic acid was estimated by the method of Winzler (1955). Fucose content was estimated by the method of Dische and Shattles (1960). Mucoproteins were estimated by the method of Winzler (1955).

Staining and quantification of MDF

The MDF staining was carried out by the method of Yoshimi et al. (2004). Briefly, the fixed colons were rinsed for 5 min in 3% acetic acid and then stained for 30 min with a solution of 1% Alcian blue pH 2.5 (Sigma Chemical Co., St. Louis, MO) in 3% acetic acid. These colons were rinsed for 10 min in 3% acetic acid to prevent nonspecific staining and then washed in distilled water. MDF was identified as focal lesions characterized by the absence or very small production of mucins under the light microscope at a low magnification. The total number and multiplicity of MDF per colon (number of crypt/MDF) were recorded.

Periodic acid and Schiff's staining

Histochemical staining of glycoconjugates was carried out as per the method of Kierman, (1990), using 2% periodic acid and Schiff's reagent in dark for 20 min. Photomicrographs were obtained using a Nikon Y-FL ECLIPSE 300 (Japan) microscope connected to a Nikon FDX-35 camera (Japan) to measure the relative intensity of PAS staining with the aid of a 40X magnification lens in control and experimental groups.

Statistical analysis

All data were analyzed with SPSS/10 Student Software. Hypothesis testing methods included one-way analysis of variance (ANOVA). The values are expressed as mean ± S.D, P value of less than 0.05 was considered to indicate statistical significance.

Results

Figure 1 shows the levels of PC, MDA and CD were summarized. These levels were increased in AOM-induced group II animals. In case of LUT treatment (group III), a significant decrease were observed. No significant changes were found in group I (control) and group IV (LUT).

Table 1 shows the incidence of MDF expression in control and experimental group of mouse. Control (group I) and LUT alone (group IV) mice showed no incidence of MDF. Induction with AOM (group II) showed increased incidence of MDF were observed when compared to control (group I). Administration of LUT (group III) reduced the incidence of MDF significantly.

Table 2 depicts the levels of glycoconjugates such

Table 1. Effect of Luteolin on the Incidence of Mucin Depleted Foci (MDF) of Control and Experimental Groups

Groups	Total no. of MDF/colon/mice	No. of Normal Like	No. of Dysplastic Crypts Foci
Control	0	0	0
AOM	14.4 ± 1.09[a]	3.7 ± 0.30[a]	10.7 ± 0.89[a]
AOM+LUT	09.2 ± 0.52[b]	1.9 ± 0.12[b]	07.3 ± 0.36[b]
LUT	0	0	0

*Each value is expressed as mean ± S.D. for six mice in each group. Statistical significance: $p<0.05$, [a]As compared with Group I, [b]As compared with Group II, ns- non significant

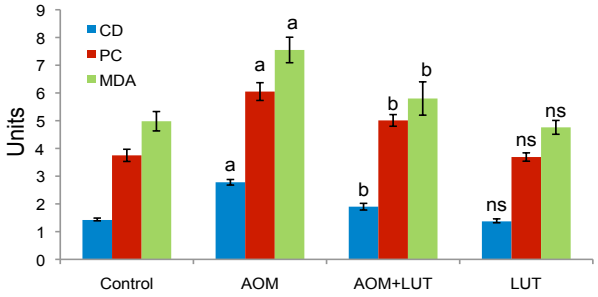

Figure 1. Effect of Luteolin on the Levels of PC, CD and MDA in Azoxymethane-Induced Colon Carcinogenesis. Values are expressed as mean ± S.D. Comparisons: Control Vs AOM and LUT, AOM Vs AOM+LUT, a P< 0.05, ns- non significant. Units- conjugated dienes-ratio of absorbance at 233 nm and 215 nm, MDA- nm/mg of protein and protein carbonyl-nmoles/mg protein

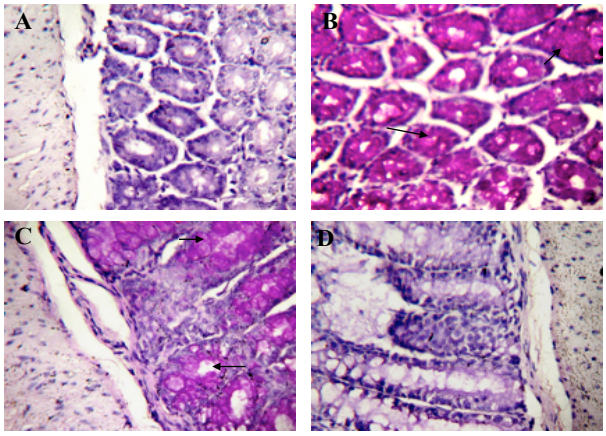

Figure 2. Histochemical Analysis by Periodic Acid Schiff's Staining in Control and Experimental Animals. The formalin fixed slides were cut in to 4μM sections then deparaffinized with xylene and rehydrated with series of alcohol. Then the slides were treated with 2% periodic acid in dark for 10 min and schiff's reagent for 2 min. Slides were then washed with distilled water and mounted. The pictures were taken at 40x magnifications. (2A) Control, (2B) AOM-induced, (2C) AOM-induced + LUT treated and (2D) LUT alone. Arrows depicts the expression of glycoconjugates

as Hexose, hexosamine, mucoprotein, sialic acid and fucose in the colon tissue of control and experimental animals. Level of glycoconjugates showed a significant ($p < 0.05$) increase in group II colon cancer-induced animals when compared with control animals. Group III (AOM+LUT) mouse showed a significant ($p < 0.05$) decrease in glycoconjugates when compared with group II. No significant changes were found between group I (control) and group IV (LUT).

Table 2 also depicts the levels of glycoconjugates such as Hexose, hexosamine, and sialic acid in the serum of control and experimental animals. Level of glycoconjugates showed a significant ($p < 0.05$) increase in group II colon cancer-induced animals when compared with control animals. Group III (AOM+LUT) mouse showed a significant ($p < 0.05$) decrease in glycoconjugates when compared with group II. No significant changes were observed between group 1 (control) and group IV (LUT).

Using the PAS method, positive reaction is detectable in the mucous cells and in the colon epithelium, suggesting that neutral glycoconjugates occur in the AOM-induced

Table 2. Parameters

Parameters	Control	AOM	AOM+LUT	LUT
Levels of glycoconjugates in colon tissues of control and experimental groups of animals:				
Hexose	1.56±0.06	4.03±0.27^a	2.99±0.16^b	1.63±0.07^{ns}
Hexosamine	0.37±0.02	1.18±0.08^a	0.74±0.04^b	0.39±0.03^{ns}
Sialic acid	0.19±0.01	0.76±0.05^a	0.43±0.03^b	0.22±0.02^{ns}
Fucose	0.16±0.01	0.69±0.04^a	0.39±0.02^b	0.17±0.01^{ns}
Mucoprotein	0.13±0.01	0.67±0.04^a	0.41±0.04^b	0.13±0.02^{ns}
Effect of Luteolin on the levels of glycoconjugates in serum of control and experimental animals:				
Hexose	242±11.15	504±41.33^a	381±28.93^b	264±11.08^{ns}
Hexosamine	33.5±1.60	61.3±4.90^a	47.1±3.38^b	34.1±1.56^{ns}
Sialic acid	44.3±1.91	82.2±7.47^a	66.0±4.50^b	45.2±1.94^{ns}

Each value is expressed as mean ± S.D. for six mice in each group. Values are expressed as mg/g of defatted tissue (colon tissue) and mg/dl (serum). Statistical significance: p<0.05, a As compared with Group I, b As compared with Group II, ns- non significant

group of mice (Figure 2A). LUT treated to colon cancer-induced mouse showed a mild decrease in glycoconjugates with degradation of tumor cells (Figure 2C). There were no significant changes were observed in control (Figure 2A) and LUT alone treated group (Figure 2D) of animals.

Discussion

MDF have been described to be pre-neoplastic lesions that can be used as a biomarker in colon carcinogenesis. MDF detected with high-iron-diamine (HID-AB) in AOM-induced rodents was recently proposed as an additional biomarker in colon carcinogenesis. However, while HID and AB (pH 2.5) stain sialomucins and sulfomucins, respectively, AB (pH 2.5) simultaneously stains both sialomucins and sulfomucins (Caderni et al., 2003). This MDF assay is simple, like ACF assay, and therefore, MDF may be useful as a biomarker of rodent colon carcinogenesis (Yoshimi et al., 2004). Our present study shows, the ratio of MDF was increased in AOM-induced mice (group II). Animals supplemented with LUT reduced the ratio of the MDF.

In the present investigation, the levels of PC, CD and MDA were found to be increased in AOM-induced animals. This may be due to enormous production of free radicals mediated process, it is involved in the formation of lipid radicals, rearrangements of unsaturated lipids that results in the formation of variety of secondary degraded products such as alkenes, MDA (Vaca et al., 1998), conjugated dienes and lipid hydroperoxides and eventually destruction of membrane lipids. Increase in the accumulation of CD in the cells can results their cellular degradation, biochemical, functional and cell death (Upsani et al., 2001). A higher level of oxidative stress, expressed as the concentration of conjugated dienes in tumor tissue, was associated with clinical progression of the tumor (Zieba et al., 2001). MDA is a mutagenic and a genotoxic agent may contribute to the development of human cancer (Ohkawa et al., 1979; Feron et al., 1991). Supplementation of LUT to the cancer bearing animals the levels of PC, CD and MDA were decreased. Lipid

peroxidation levels were increased in colon cancer status (Skrzydlewska et al., 2003). Luteolin administration restored the lipid peroxidation levels, which may be due to the anti-oxidant activity of luteolin (Samy et al., 2006; Ashokkumar & Sudhandiran, 2008).

Glycoproteins play crucial role in mediating cell surface function, such as cell–cell recognition, cellular adhesion, binding and clearance of serum glycoproteins and metabolic transport among others. Increased levels of glycoprotein contents are valuable indicators of carcinogenic process and these changes alter the rigidity of cell membrane (Selvam & Nagini, 1995). Malignant transformation of normal cell may be accompanied by changes in the carbohydrate composition of glycoproteins viz. hexose, hexosamine and sialic acid in plasma membrane. Elevated levels of protein-bound glycoconjugates such as hexose, hexosamine and sialic acid in plasma of various cancers were reported (Patel et al., 1989). It has been reported that elevated levels of glycoconjugates in serum of cancer patients are due to release of glycoconjugates from the cell membrane to the serum, undergo increased turnover in cancer patients (Hickey et al., 1986). Evidence from animal studies suggested that, the presence of malignant tumors invoke increased hepatic synthesis of glycoproteins (Macbeth & Bekesi, 1964).

We have observed that, increased levels of glycoprotein such as hexose, hexosamine, fucose, mucoprotein and sialic acid in serum and colon tissues of AOM-induced mouse. Altered levels of protein bound carbohydrate are well documented during neoplastic diseases (Shelter et al., 1950; Srinivasan et al., 2006). These reported changes in surface carbohydrates during cellular differentiation and neoplastic transformation suggest their importance in physiology and behaviour of the cells. Such changes have long been implicated in malignant transformation (Hynes, 1978). Elevations of glycoprotein components serve as a classical marker and as an indicator in the progression of tumor growth. The crucial roles of cell surface and membrane constituents in neoplastic behavior and changes in normal serum glycoconjugates have long been associated with malignancies (Patel et al., 1990). Sialic acid is an acylated derivative of neuraminic acid and exists as a terminal component of the non-reducing end of carbohydrate chains of glycoprotein in mammals. Their implications in a variety of surface-related vital cell functions in numerous tissues are well documented (Olden et al., 1982). Fucose has hydrophobic properties (Montreuil, 1980) and its presence in the mucous gland secretion and in the epidermis interstices can represent a barrier against the animal desiccation. In the present investigation, administration of LUT brought back the glycoconjugates to near normal levels. Flavonoids such as epigallo-catechin-3gallate (EGCG), has the ability to reduce the levels of glycoconjugates in the tumor condition (Srinivasan et al., 2006).

Glycoprotein levels are high in tumor tissue due to increased lipid peroxidation resulting in lowered antioxidant status (Scholz et al., 1979), which was in concordance with our present study, the levels of LPO was increased in AOM-induced animals (Ashokkumar & Sudhandiran, 2008). Treatment with strong antioxidants reduced the levels of glycoconjugates (Srinivasan et al., 2006). Samy et al. (2006) reported that treatment with luteolin decreased the levels of oxidative stress by scavenging lipid peroxides and enhanced the activities of antioxidant enzymes. Luteolin also a potent antioxidant (Perez-Garcia et al., 2000) and reduced the levels of LPO.

Hence from the above findings, LUT may alter cell membrane glycoprotein synthesis and structure, indicating its potent antioxidant property. This reduction in the levels of glycoprotein components indicates that luteolin has the ability to suppress malignancy by modulating cell transformation, decreasing the degree of colon cancer growth and controlling cell proliferation.

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