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Original Article

Protective effect of coumarin on cell surface glycoconjugates abnormalities during 7,12-dimethylbenz(a)anthracene (DMBA) induced oral carcinogenesis

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ABSTRACT

Aims: The major aims of the present study was to focus the protective effect of coumarin on cell surface glycoconjugates abnormalities during 7,12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Methods: DMBA painting in the hamster buccal pouch three times per week for 14 weeks developed oral squamous cell carcinoma. The levels of glycoconjugates (protein bound hexose, hexosamine, sialic acid and fucose) were analyzed by using specific colorimetric methods. Results: An increase in glycoconjugates was noticed in the plasma and buccal mucosa of oral cancer bearing golden Syrian hamsters. Coumarin administration orally at a dose of 100mg/kg b.w to hamsters treated with DMBA brought back the status of glycoconjugates to near normal range. Conclusion: Present study thus focused the protective effect of coumarin on cell surface abnormalities occurring during DMBA-induced hamster buccal pouch carcinogenesis.

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1. Introduction

Oral cancer is one of the most common cancer worldwide and a predominant cancer in India. This form of cancer accounts for 40-50% of all cancers in India. Indian peoples facing formidable health problem and highest morbidity and mortality due to oral cancer [1]. Long term habits of tobacco chewing, smoking, betel quid chewing and alcohol consumption are strongly attributed to oral carcinogenesis [2]. Golden Syrian hamsters are commonly used for oral cancer studies as an ideal model due to their buccal pouch (pocket like anatomy), which can able to retain the carcinogen such as DMBA for longer time upon treatment. Moreover, the tumor developed in the hamster buccal pouch has many similarities with human oral tumor, morphologically, biochemically and at molecular level [3].

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Glycoproteins, a family of complex proteins, are the vital components of the cell surface. The cell surface glycoproteins not only play a crucial role in cell differentiation and intercellular recognition but also play an important role during neoplastic transformation [4]. Analysis of plasma and tissue glycoproteins levels could provide valuable information in establishing diagnosis, determining clinical tumor staging and detecting metastasis. Glycoconjugates status in plasma or tissues can also be used as a biomarker in the treatment monitoring and management of cancer patients [5].

Many properties of mammalian cells are either expressed at or mediated through the cell surface. The neoplastic transformation is often associated with profound alterations in the cell-membrane glycosylation. It has been reported that aberrant glycosylation of glycoconjugates is the major molecular changes that accompany neoplastic transformation [6]. Sialic acid, a monosaccharide with nine carbon backbone and fucose, a sugar abundant in mother's milk, are the most important and principal terminal sugars of glycoproteins. They have crucial role in many physiological and pathological processes. Increased sialylation of cell surface glycoconjugates is among the key molecular changes associated

with malignant transformation and cancer progression [7]. Dabelsteen et al [8] suggested that tumor associated glycoconjugates status can be used in the diagnosis of human cancers. Human body needs fucose as one of the essential sugar for optimum cell-to-cell communication. Increased levels of sialic acid and fucose were reported in the plasma and tumor tissues of patients with several forms of cancers including oral cancer [9].

Coumarin [1,2-benzopyrone (fig 1)] is an active constituent of several plants (Cassia, lavender, yellow sweet clover and woodruff) and fruits (bilberry and cloudberry). It also occurs in green tea and other foods such as chicory. Profound studies demonstrated diverse pharmacological and biochemical effects of coumarin, which include anti-bacterial, anti-viral, anti-mutagenic, anti-diabetic, anti-cancer and antioxidant properties [10, 11]. Previous studies from our laboratory have demonstrated the antigenotoxic potential of coumarin in DMBA-induced genotoxicity and chemopreventive potential of coumarin against DMBA-induced oral carcinogenesis [12] . Present study was designed to focus the protective effect of coumarin on cell surface abnormalities by measuring the status of glycoconjugates during DMBA-induced oral carcinogenesis in golden Syrian hamsters.

2. Materials and methods

2.1. Chemicals

DMBA and coumarin were purchased from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade, purchased from Hi-media Laboratories, Mumbai, India.

2.2. Animals

Male golden Syrian hamsters, aged 8–10 weeks, weighing 80–120 g, were obtained from the National Institute of Nutrition, Hyderabad, India and were maintained in the Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages at room temperatures (27±2 °C) with relative humidity 55±5%, in an experimental room. The local institutional animal ethics committee (Registration Number 160/1999/CPCSEA), Annamalai University, Annamalainagar, India approved the experimental design (Proposal No.731, dated 02.09.2010). The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with the Indian National Law on animal care and use. The animals were provided with standard pellet diet (Amrut Laboratory Animal Feed, Mysore Feeds Limited, Bangalore, India) and water ad *libitum.*

2.3. Experimental design

A total number of 40 hamsters were randomized into four groups and each group contained 10 hamsters. Group I animals served as the control and were treated with liquid paraffin (vehicle) alone three times a week for 14 weeks on their left buccal pouches. Group II animals were treated with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group II animals received no other treatment. Group III animals were treated with DMBA as in group II, received in addition oral administration of coumarin at a dose of 100 mg/kg b.w/day, starting 1 week before exposure to the carcinogen and it should be continued on alternate days to DMBA painting until the animals were sacrificed. Group IV animals received oral administration of coumarin alone, as in group III, throughout the experimental period. The experiment was terminated at the end of 16th week and all animals were sacrificed by cervical dislocation.

2.4. Histopathology

For histopathological examination, buccal mucosa were fixed in 10% formalin and routinely processed and embedded with paraffin, 2–3 µm sections were used for histological studies. For detection of glycoconjugates, the tissue sections of buccal mucosa were immersed in a solution of 0.1% periodic acid for 15 minutes, at 50°C. The slides were washed in running tap water and immersed in Schiff's reagent for 40 minutes. Subsequently, the sections were washed in running tap water for 10 minutes, counterstained with hematoxylin, dehydrated in graded ethanol, cleared in xylene and mounted in resinous medium.

2.5. Biochemical studies

Biochemical studies were conducted in the plasma and buccal mucosa of golden Syrian hamsters. The precipitate obtained after treating the plasma with 95 % ethanol was used for the estimation of protein bound hexose and hexosamine. The defatted tissues obtained after treating buccal mucosa with methanol and chloroform was used for the estimation of glycoproteins. To the dry defatted tissues remaining after lipid extraction, 0.1N H_2SO_4 was added and hydrolyzed at 80°C for 1h. It was cooled and the aliquot was used for sialic acid estimation. To the remaining solution, 0.1N sodium hydroxide was added and kept in an ice bath for 1 h. From these aliquots, protein bound hexose and fucose were estimated. The protein bound hexose, hexosamine, total sialic acid and fucose were estimated by the methods of Niebes [13], Wagner [14], Warren [15] and Dische and Shettles [16] respectively.

2.6. Statistical analysis

The data are expressed as mean ± SD. Statistical comparisons were performed by one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant if the p values were 0.05 or less.

3. Results

Table 1 and 2 shows the levels of glycoconjugates in the plasma (protein bound hexose, hexosamine, total sialic acid and fucose) and buccal mucosa (protein bound hexose, total sialic acid and fucose) glycoconjugates of control and experimental hamsters in each group. The levels of glycoconjugates in the plasma and buccal mucosa were significantly increased in hamsters treated with DMBA alone (group 2) as compared to control hamsters (group 1). Oral administration of coumarin to DMBA treated hamsters (group 3) brought back the levels of above said glycoconjugates to near normal range. No significant difference was noticed in the levels of plasma and buccal mucosa glycoconjugates in coumarin alone (group 4) treated hamsters as compared to control hamsters (group 1).

Table 1. Status of plasma glycoconjugates (protein bound hexose, hexosamine, total sialic acid and fucose) in control and experimental hamsters in each group

Parameters	Protein bound Hexose (mg/dl)	Protein bound hexosamine (mg/dl)	Total sialic acid (mg/dl)	Fucose (mg/dl)
Control	88.13 ± 7.61[a]	77.81 ± 6.37[a]	44.98 ± 3.67[a]	7.22 ± 0.54[a]
DMBA	135.83 ± 12.07[b]	118.21 ± 9.76[b]	81.22 ± 7.18[b]	15.37 ± 1.42[b]
DMBA + Coumarin	96.58 ± 8.82[c]	86.12 ± 7.23[c]	50.13 ± 4.57[c]	8.03 ± 0.81[c]
Coumarin alone	87.71 ± 7.92[a]	78.01 ± 6.11[a]	45.02 ± 4.11[a]	7.31 ± 0.68[a]

Values are expressed as mean ± SD (n=10). Values that are not sharing a common superscript in the same column differ significantly at p<0.05.

Table 2. Status of buccal mucosa glycoconjugates (protein bound hexose, total sialic acid and fucose) in control and experimental hamsters in each group.

Parameters	Protein bound Hexose (mg/dl)	Protein bound hexosamine (mg/dl)	Total sialic acid (mg/dl)
Control	107.84 ± 9.93[a]	107.84 ± 9.93[a]	14.11 ± 1.22[a]
DMBA	152.61 ± 14.62[b]	152.61 ± 14.62[b]	31.73 ± 2.76[b]
DMBA + Coumarin	119.70 ± 11.47[c]	119.70 ± 11.47[c]	16.01 ± 1.84[c]
Coumarin alone	107.97 ± 9.88[a]	107.97 ± 9.88[a]	14.56 ± 1.08[a]

Values are expressed as mean ± SD (n=10). Values that are not sharing a common superscript in the same column differ significantly at p<0.05.

Figure 2 (a - d) shows glycoconjugates expression pattern in the buccal mucosa of control and experimental hamsters in each group. The glycoconjugates expression pattern was analysed using periodic acid Schiff's staining in the buccal mucosa. We observed increased glycoconjugates expression in the buccal mucosa of tumor bearing hamsters (group 2; figure. 2b). Oral administration of coumarin to DMBA treated hamsters (group 3; figure 2c) significantly reduced the expression of glycoconjugates in the buccal mucosa. Glycoconjugates expression pattern was similar in coumarin alone treated (group 4; figure 2d) and control (group 1; figure 2a) hamsters.

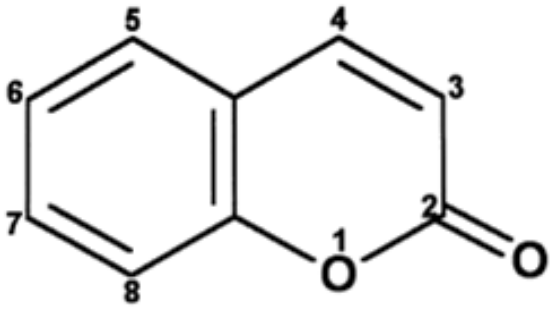
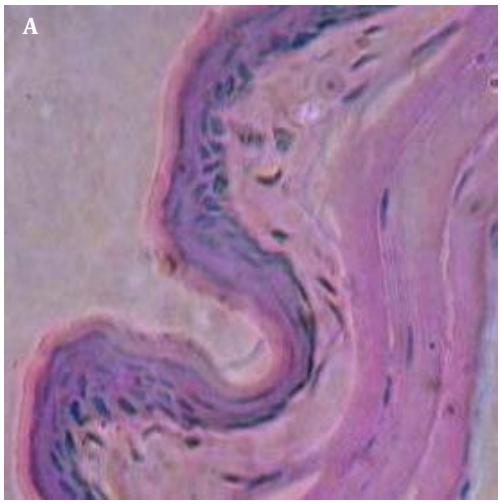


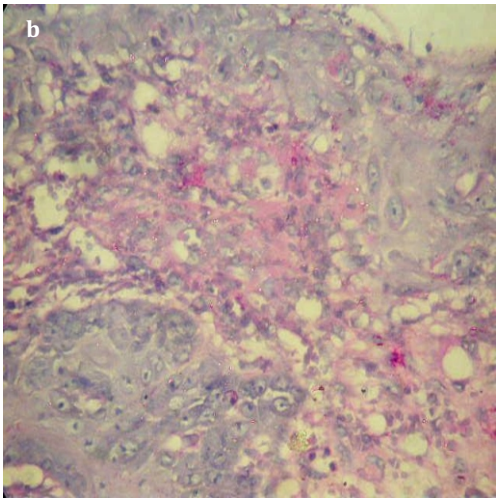
Fig 1. Chemical Structure of coumarin

Fig 2.(a - d) Glycoconjugates expression pattern in the buccal mucosa of control and experimental animals in each group.

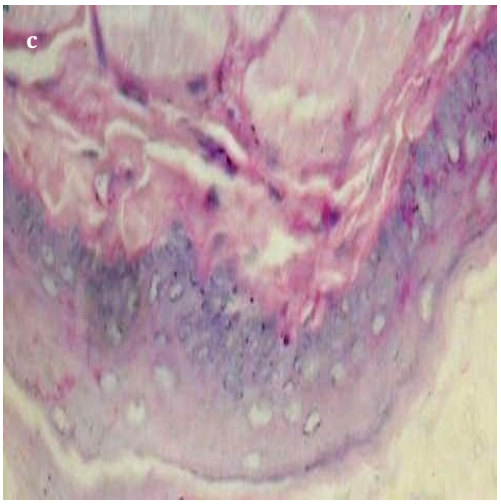


a) Normal glycoconjugates expression in the control hamsters (40X)

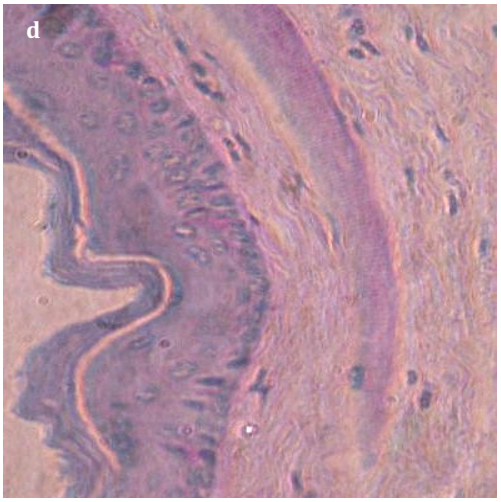
(b) Over expression of glycoconjugates in hamsters treated with DMBA alone (40X)



(c) Lowered expression of glycoconjugates in DMBA + coumarin treated hamsters (40X)



(d) Normal glycoconjugates expression in hamsters treated with coumarin alone (40X)



4. Discussion

In recent years, considerable researches have been carried out on the status of glycoconjugates in cancerous conditions due to the reason that glycoconjugates play a vital role during malignant transformation. Cell surface glycoconjugates, the major determinants of cell properties and function, undergo abnormal expression due to aberrant glycosylation in cancerous conditions [17]. Aberrant glycosylation is the key feature involved in the conversion of normal cell into a malignant one. Atypical glycosylation and degradation of cell surface carbohydrates have been shown in oral carcinogenesis [18]. Over expression of glycoconjugates in the cell surface of carcinogen treated experimental animals has been reported [19]. A large number of experimental studies pointed out that glycoproteins were synthesized enormously in the tumor and liver tissues during cancerous conditions and subsequently entered into circulation [20]. Over expression of glycoconjugates in the tumor cells with subsequent shedding into plasma could account for increased levels of plasma protein bound hexose, hexosamine, sialic acid and fucose.

Studies have shown a selective increase in existing specific sialylated sequence or tumor associated denovo synthesis of specific sialylated sequences. Two fold increases in the plasma or tissue sialic acid content in tumor bearing animals has been reported [21]. Elevated levels of fucose, a terminal pentose sugar of glycoprotein chain, has been reported in the plasma of patients with various types of malignancies [22-24]. Elevated fucose content due to abnormal fucosylation has been reported in several cancers [21, 25]. It has been suggested that elevated levels of sialic acid and fucose in the cell surface may facilitate tumor metastasis. Enhanced fucosylation in the tumor cell surface could contribute to decreased adhesion and uncontrolled proliferation. Elevated serum fucosidase activity has been reported in oral pre-cancer and oral cancer patients [26]. Tomsik et al have demonstrated the anticancer potential of L-fucose [27]. Profound studies on experimental and human oral carcinogenesis concluded that sialic acids and fucose were increased in the plasma due to increased turn over and shedding from rapidly proliferating tumor cells [5, 28]. Our results are in line with these findings.

Oral administration of coumarin to hamsters treated with DMBA significantly reduced the levels of plasma and buccal mucosa glycoconjugates. Histopathological assessment of glycoconjugates expression pattern in the experimental animals coincides with the biochemical findings, supports the protective effect of coumarin on DMBA-induced cell surface abnormalities.

5. Conclusion

Our results thus suggest that coumarin has considerable potential to protect the cell surface glycoconjugates moieties during DMBA-induced oral carcinogenesis. The protective effect of coumarin is probably due to its suppressive effect on glycoprotein synthesis by modulating the activities of the enzymes involved in the glycosylation. Further studies are therefore be warranted to assess the coumarin efficacy on the activities of enzymes involved in the process of glycosylation.

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