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Research Article

Modulating effect of Andrographolide on cell surface glycoconjugates status during 7,12-dimethylbenz(a) anthracene induced hamster buccal pouch carcinogenesis

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ABSTRACT

Cell surface glycoconjugates play an important role in pinocytosis, cell differentiation, intra cellular recognition, cell adhesion and tumorigenesis. Aim of the present study was to investigate the protective effect of andrographolide on cellular integrity by measuring the status of glycoconjugates in the buccal mucosa and plasma of hamsters during 7,12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Oral squamous cell carcinoma was developed in the buccal pouch of hamsters by painting with DMBA three times a week for 14 weeks. The status of glycoconjugates was assayed in plasma and buccal mucosa using the specific calorimetric methods. We noticed 100% tumours formation with marked abnormalities in the status of plasma and buccal mucosa glycoconjugates in hamsters treated with DMBA alone. Oral administration of andrographolide at a dose of 50 mg/kg bw not only completely prevents the tumour formation, but also restored the status of glycoconjugates in DMBA treated hamsters. The present study thus demonstrates the protective effect of andrographolide on cell surface glycoconjugates abnormalities during DMBA-induced hamster buccal pouch carcinogenesis.

Keywords: Oral Cancer; Glycoconjugates; DMBA; Andrographolide

INTRODUCTION

Oral squamous cell carcinoma is responsible for high rate of morbidity and mortality throughout the world. Oral cancer is the fifth most common malignancy worldwide and affects 500,000 new cases each year (Warnakulasuriya S. 2009). Epidemiological studies pointed out that the annual incidence of oral cancer is higher in developing countries including India. Tobacco and betel quid chewing, smoking and alcohol consumption are mainly attributed to the pathogenesis of oral cancer (Petti S. 2009). 7,12-dimethylbenz(a)anthracene (DMBA) is a potent and well known pro-carcinogen, is commonly used to induce oral cancer in experimental animals. DMBA-induced oral carcinogenesis in golden Syrian hamsters closely mimics the human oral tumour, histologically, biochemically and at molecular level (Manoharan S et al. 2010). DMBA-induced oral cancer thus serves as an ideal model to investigate the abnormalities in cell surface glycoconjugates in the circulation and buccal mucosa of golden Syrian hamsters.

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Glycoconjugates, the essential components of biological organisms, play a vital role in cell-cell communication, cell differentiation, cell adhesion and as receptors for many hormones (Dabelsteen E. 1996). The structural glycoproteins and proteoglycans dominate cellular and biochemical events in the tissues. The role of glycoconjugates in tumorigenesis is well known. Abnormal glycosylation pattern has been documented well in several cancerous conditions including oral cancer. Glycoconjugates are used as biomarkers of diagnosis and prognosis of several cancers including oral cancer (Thiery JP and Ovtracht LD, 1979). Sialic acid, a terminal nine-carbon sugar of glycoconjugates moieties, play important role in cell adhesion and antigenicity (Sonmez H et al. 1999). Fucose is utilized as an essential sugar for optimal function of cell-cell communication and has crucial role in cancer and its spread (Schutter EM et al. 1992). Extensive studies demonstrated increased levels of glycoproteins, sialic acid and fucose in serum and tissues of tumour bearing animals and patients with cancer.

Andrographolide, a diterpene lactone, is one of the major bioactive constituents of *Andrographis paniculata* (Rajani M et al. 2000; Lomlim L et al. 2003). Andrographolide possesses wide range of biological and pharmacological effects including anticancer, anti-inflammatory, antioxidant and cardioprotective properties (Mishra SK et al.2007; Patarapanich C et al. 2007). Previous studies from our laboratory demon-

strate the antigenotoxic potential of andrographolide against DMBA-induced genotoxicity (Manoharan S et al. 2011). The present study focuses the protective effect of andrographolide on cell surface abnormalities during DMBA-induced hamsters buccal pouch carcinogenesis.

MATERIALS AND METHODS

Chemicals

The carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA), and andrographolide was obtained from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade, and were purchased from HiMedia Laboratories, Mumbai, India.

Animals

Forty male golden Syrian hamsters, 8 weeks old, weighing 80-120g, were obtained from National Institute of Nutrition, Hyderabad, India and maintained in Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages and provided standard pellet diet and water *ad libitum*. The animals were maintained under controlled conditions of temperature and humidity with a 12 h light- dark cycle.

EXPERIMENTAL DESIGN

The local institutional animal ethics committee (Registration number 160/1999/ CPCSEA) of Annamalai University approved the experimental design. Animals were maintained in accordance with the guidelines of ethical committee for animal care of Annamalai University in accordance with Indian National Law on animal care and use. A total of 40 hamsters were randomized into four groups of ten animals in each. Group I animals served as control and were painted with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Groups II and III animals were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group II animals received no further treatment. Group III animals were orally given andrographolide at a dose of 50 mg/kg bw/day, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the sacrifice of the animals. Group IV animals received oral administration of andrographolide alone throughout the experimental period. The experiment was terminated at the end of 16 weeks and all animals were sacrificed by cervical dislocation. Biochemical studies were conducted on plasma, liver and buccal mucosa of control and experimental animals in each group.

Histological studies

Histological investigations were performed on buccal mucosal tissues of the control and experimental animals. Tissues were fixed in 10% buffered formalin and routinely processed and embedded with paraffin, 2-3µm 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 50ºC. 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.

Biochemical Analysis

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 glycoprotein. 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 1h. 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 P (1972), Wagner *et al.* (1979), Warren *et al.* (1959), and Dische and Shettles, (1948) respectively.

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.

RESULTS

The tumor incidence, tumor volume and tumor burden of control and experimental hamsters in each group are shown in Table 1. We have observed 100% tumor formation with mean tumor volume (329.18mm^3) and tumor burden (1020.45mm^3) in DMBA alone treated hamsters. Oral administration of andrographolide at a dose of 50mg/kg body weight completely prevented the tumor incidence, tumor volume and tumor burden in DMBA treated hamsters. No tumor was observed in control hamsters painted with liquid paraffin alone as well as andrographolide alone administered hamsters.

Figure 1(A-D) and Figure 2(A-C) show the levels of glycoconjugates in plasma (protein bound hexose, hexosamine, total sialic acid and fucose) and buccal mucosa glycoconjugates (protein bound hexose, total sialic acid and fucose) of control and experimental hamsters in each group. The levels of glycoconjugates in plasma and buccal mucosa were significantly increased in DMBA alone painted hamsters (group 2) as compared to control hamsters (group 1). Oral administration of andrographolide to DMBA treated hamsters (group 3) brought back the levels of above said glycoconjugates

Table 1: Incidence of oral neoplasm in control and experimental hamsters in each group (n = 10)

Parameter	Group I (Control)	Group II (DMBA alone)	Group III (DMBA + andrographolide)	Group IV (andrographolide alone)
Tumor incidence (oral squamous cell carcinoma)	0	100%	0	0
Total number of tumour / animals	0	31/10	0	0
Tumour volume (mm^3)/animals	0	329.18 ± 29.72	0	0
Tumour burden (mm^3)/animals	0	1020.45 ± 92.13	0	0

Tumor volume was measured using the formula, $v=(4/3)\pi(D_1/2)(D_2/2)(D_3/2)$ where D_1, D_2 and D_3 are the three diameters (mm) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors/animal.

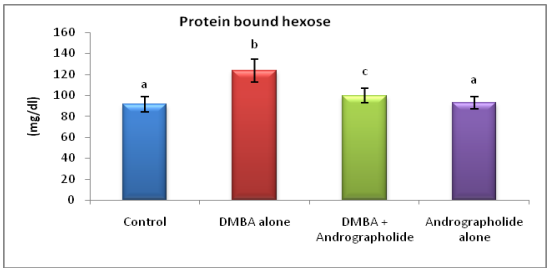

Figure 1A.

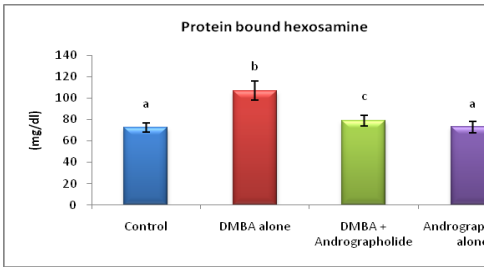

Figure 1B.

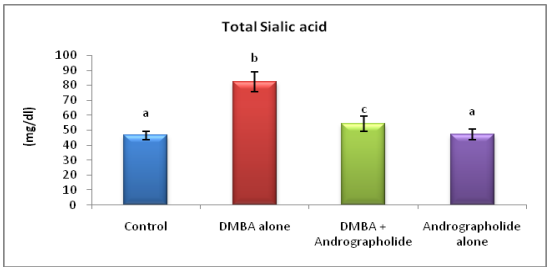

Figure 1C.

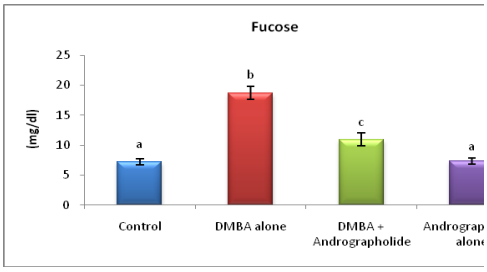

Figure 1D.

Figure 1: Status of glycoconjugates in plasma of control and experimental hamsters in each group

Figure 1(A-D) shows the levels of glycoconjugates in plasma [protein bound hexose (1A), hexosamine (1B), total sialic acid (1C) and fucose (1D)] of control and experimental hamsters in each group. Values are expressed as mean ± SD (n=10). Values that are not sharing a common superscript differ significantly at $p<0.05$.

to near normal range. No significant difference was noticed in the levels of plasma and buccal mucosa glycoconjugates in andrographolide alone (group 4) treated hamsters as compared to control hamsters (group 1).

Figure 3 (A-D) shows glycoconjugates expression pattern in the buccal mucosa of control and experimental hamsters in each group. The glycoconjugates expression pattern was analyzed using periodic acid Schiff's staining in the buccal mucosa. We observed increased glycoconjugates expression in the buccal mucosa of the tumour bearing hamsters (group 2; figure 3B). Oral administration of andrographolide to DMBA treated hamsters (group 3; figure 3C) significantly reduced the expression of glycoconjugates in the buccal mucosa. Glycoconjugates expression pattern was similar in andrographolide alone treated (group 4; figure 3D) and control (group 1; figure 3A) hamsters.

Figure 1(A-D) and Figure 2(A-C) show the levels of glycoconjugates in plasma (protein bound hexose, hexosamine, total sialic acid and fucose) and buccal mucosa glycoconjugates (protein bound hexose, total sialic acid and fucose) of control and experimental hamsters in each group. The levels of glycoconjugates in plasma and buccal mucosa were significantly increased in DMBA alone painted hamsters (group 2) as compared to control hamsters (group 1). Oral administration of andrographolide 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 andrographolide alone (group 4)

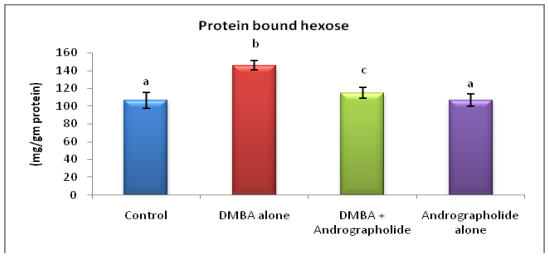

Figure 2A.

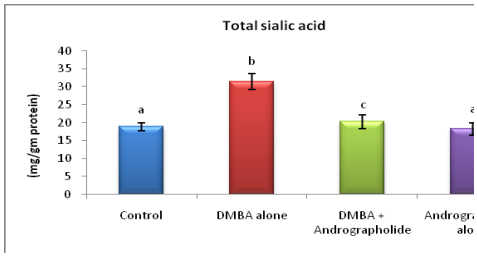

Figure 2B.

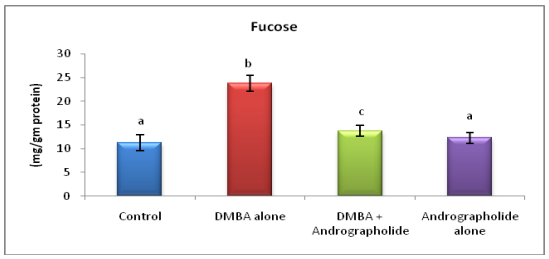

Figure 2C.

Figure 2: Status of glycoconjugates in buccal mucosa of control and experimental hamsters in each group

Figure 2 (A-C) shows the levels of glycoconjugates in buccal mucosa [protein bound hexose (2A), total sialic acid (2B) and fucose (2C)] of control and experimental hamsters in each group. Values are expressed as mean ± SD (n=10). Values that are not sharing a common superscript differ significantly at $p<0.05$.

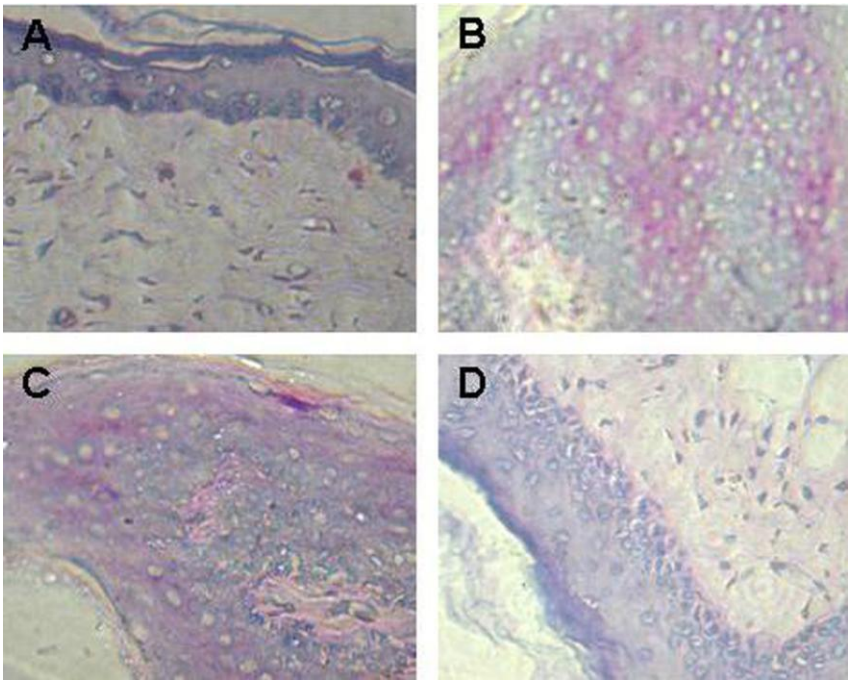

Figure 3: Glycoconjugates expression pattern in the buccal mucosa of control and experimental hamsters in each group

Figure 3 (A-D) shows the glycoconjugates expression pattern in the buccal mucosa of control and experimental animal in each group. (3A) Normal glycoconjugates expression in the control hamsters (40X), (3B) Over expression of glycoconjugates in hamsters treated with DMBA alone (40X), (3C) Lowered expression of glycoconjugates in DMBA + Andrographolide treated hamsters (40X), (3D) Normal glycoconjugates expression in hamsters treated with andrographolide alone (40X).

treated hamsters as compared to control hamsters (group 1).

Figure 3 (A-D) shows glycoconjugates expression pattern in the buccal mucosa of control and experimental hamsters in each group. The glycoconjugates expression pattern was analyzed using periodic acid Schiff's staining in the buccal mucosa. We observed increased glycoconjugates expression in the buccal mucosa of the tumour bearing hamsters (group 2; figure 3B). Oral administration of andrographolide to DMBA treated hamsters (group 3; figure 3C) significantly reduced the

expression of glycoconjugates in the buccal mucosa. Glycoconjugates expression pattern was similar in andrographolide alone treated (group 4; figure 3D) and control (group 1; figure 3A) hamsters.

DISCUSSION

In the present study, oral administration of andrographolide at a dose of 50 mg/kg bw completely prevented the formation of tumours in the buccal pouch of hamsters treated with DMBA. The results of the present study suggest the anti-tumour initiating potential of andrographolide during DMBA-induced hamster buccal pouch carcinogenesis. In the present study, increase in buccal mucosa glycoconjugates accompanied by an increase in plasma glycoconjugates was noticed in hamsters treated with DMBA alone. Alterations in the cell surface glycoconjugates could result in abnormal cell growth metastasis and changes in cell-adhesion (Snyder HB et al. 2002). Atypical glycosylation of cell surface carbohydrates could result in malignant transformation of oral epithelium. Enhanced activity of β-D galactosidase has been reported in experimental oral carcinogenesis (Brooks SA et al. 2000). A partial loss of glycoconjugates from epithelial cells has been reported in oral cancer (Manoharan S et al. 2004). Increase in tumour tissue protein bound hexose and fucose has been reported in several cancers including oral cancer (Srinivasan P et al. 2006). It has been reported that tumour cells synthesize glycoproteins during cancerous conditions (Aranganathan S et al. 2005). A large number of experimental studies demonstrated shedding of glycoconjugates from tumour tissues into circulation, which accounts for increased plasmic glycoconjugates in tumour bearing animals (Feijoo C et al. 1997). Our results corroborate this observation.

The status of sialic acid in plasma may be considered as a useful index for monitoring the tumour staging as well as therapeutic response. Over accumulation of sialic acid content in both plasma and tumour tissues has been shown in human and experimental oral cancer (Shashikanth MC, Rao BB, 1994; Sanjay PR et al. 2008). Tumors cell utilize their higher sialic acid and fucose content in the cell surface as a mask to avoid recognition by the immune surveillance systems which facilitate tumor metastasis (Narayanan S. 1994). Our results are in line with these findings. Fucose, the only monosaccharide, present in L-con formation, occupies terminal position and not inserted in the oligosaccharide chains of vertebrate glycoconjugates (Flowers HM.1981). Increased levels of fucose were repoted in several malignancies including oral cancer (Patel PS et al. 1994; Wang JW et al. 1995). The fucose content increases gradually in the tumour tissues from precancerous lesions to advanced malignancy (MacDougall SL et al. 1987). Aberrant fucosylation is one of the major characteristic features of malignant tumours (Alhadeff JA. 1989; Hakomori SI. 1989). It has been reported that increased turnover and secretion of sialic acid and fucose in tumour tissues may induce their shedding into circulation, which accounts for increased levels of plasma sialic acid and fucose (Varazin G et al. 1990).

Oral administration of andrographolide restored the status of protein bound hexose, protein bound hexosamine, sialic acid and fucose in both plasma and buccal mucosa of hamsters treated with DMBA. Our results suggest that andrographolide protected the cell surface abnormalities during DMBA induced hamster buccal pouch carcinogenesis. Increase in glycoconjugates in tumour tissues were also confirmed in this study with the help of histological studies using periodic acid Schiff's base (PAS) staining.

CONCLUSION

Although the exact mechanism has not been elucidated for the protective effect of andrographolide on cell surface abnormalities, the possible mechanism may include its inhibitory effect on enzymes involved in the synthesis of glycoconjugates. Further studies are thus warranted to study the effect of andrographolide on the activities of enzymes involved in glycosylation, sialylation and fucosylation process.

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