Impact of naringenin on glycoprotein levels in *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced gastric carcinogenesis in rats

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We have studied the chemopreventive role of naringenin against experimental gastric carcinogenesis in male Wistar rats. The animals were divided into five groups and six animals were included in each group. Stomach, liver, sera and kidney specimens were collected in the 20th week and the level of glycoproteins namely, hexose, hexosamine, sialic acid and fucose, were measured in the control, gastric cancer-induced, cancer naringenin pretreated, cancer naringenin posttreated and naringenin alone animals. The glycoprotein levels were increased in the gastric cancer-induced rats when compared with the control rats. The levels of glycoprotein were decreased significantly in cancer-bearing rats supplemented with naringenin as compared with the gastric cancer-induced rats. The result shows the gastroprotective effect of naringenin and describes the likelihood of naringenin in maintaining the integrity of cell membranes and gastric mucosa against oxidative damage. Moreover, we hypothesize that regulation of glycoprotein levels by naringenin could be associated with the regression of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced gastric carcinoma. *Anti-Cancer Drugs* 19:885–890 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Gastric cancer ranks as the second most common (10.4%) cause of cancer deaths worldwide [1,2]. Owing to its high incidence, marked attention is being given to prevent gastric cancer at the earliest possible stage. Experimental evidence suggests that sodium chloride could markedly augment the carcinogenic effects of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in the glandular stomach [3]. Sodium chloride decreases the viscosity of gastric mucin and disconcerts the mucosal integrity. Although a high salt diet decreases the amount of glandular mucin (which acts against *Helicobacter pylori* infection), recent experimental data suggest that salt could increase the risk of stomach cancer by several folds [4].

Glycoproteins (GPs) play a crucial role in the maintenance of cellular phenomena during cancerous transformation. GP levels are high in tumour tissues because of increased lipid peroxidation, which results in lowered antioxidant status [5], aberrant glycosylation [6] and increased lysosomal hydrolases and proteases [7]. It is widely believed that the carbohydrate moieties of GPs notably hexose, hexosamine, fucose and sialic acid have an important role in protein stability and functions [8]. Glycosidase distribution in serum has been documented in mammalian systems [9,10]. GPs are frequently used as diagnostic and prognostic markers of squamous cell carcinoma [11]. Elevated levels of glycosidases may occur in fibroblasts that are transformed by oncogenic viruses [12].

Of the various modern approaches undertaken to curtail the incidence of cancer, chemoprevention has been in the limelight in recent years. Fruits, vegetables and herbal beverages with diverse pharmacological properties have been found to possess a myriad of cancer prevention potentials [13–17]. It is widely believed that carcinogenesis can effectively be kept at bay by the ingestion of certain plant-derived foods rich in flavonoids [18–21]. Naringenin, a natural flavanone of citrus fruits, has a plethora of pharmacological properties, that is, anticarcinogenic, antimutagenic, anti-inflammatory and antiatherogenic potentials [22–26]. Recent evidence suggests that naringenin can effectively scavenge free radicals and reactive oxygen species both *in vivo* and *in vitro* [27]. Therefore, we have measured GPs as a measure of gastric carcinogenesis and determined the role of naringenin against MNNG-induced gastric carcinogenesis in experimental rats.

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Materials and methods

Animals and diet

Male Wistar albino rats aged 6 weeks, procured from the Central Animal House Facility of the University of Madras were used in the experimental study. The animals were fed with normal rat chow (Hindustan Lever Limited, Mumbai, India) and clean drinking water *ad libitum*. The rats were maintained under standard temperature and humidity with an alternating 12-h light–dark cycle. Briefly, the animals were maintained according to the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the Animal Ethics Committee of University of Madras (02/055/05).

Chemicals and reagents

The following chemicals were purchased from the indicated sources: MNNG, naringenin and bovine serum albumin (Fluka-Chemika-Biochemika, Buchs, Switzerland; Sigma Chemical Co., St Louis, Missouri, USA). Thiobarbituric acid, trichloroacetic acid, Ehrlich's reagent and solvents used were of analytical grade procured from SD Fine Chemicals and Sisco Research Labs, Mumbai, India.

Experimental procedures

The experimental rats were randomly divided into five groups; each group consisted of six animals. Animals in group I were fed a normal diet and corn oil (vehicle). Groups II–IV received MNNG in the drinking water for 4 weeks (three times a week for weeks 1 and 3, 200 mg/kg body weight). Rats in group III were given naringenin (200 mg/kg body weight in corn oil) starting week 1 before MNNG exposure until the end of week 8 and were maintained for 20 weeks. Group IV rats were treated with naringenin (200 mg/kg body weight in corn oil) from week 8 after MNNG induction. Group V animals were never exposed to MNNG; they received naringenin throughout the experimental period. The experiment was completed at the end of week 20, and all the rats were killed with ether anaesthesia. Stomach, liver and kidney tissues were excised and blood was collected with anticoagulant, plasma was separated and used for GP investigations and other biochemical analyses. Protein estimation was done by the method of Lowry *et al.* [28].

Glycoprotein estimation

The tissues excised were defatted [29], treated with 0.05 mol/l H_2SO_4 and hydrolyzed at 80°C. The resulting aliquot was used for sialic acid estimation. NaOH of 0.1 mol/l was added to the remaining solution. The aliquots were used for fucose, hexose and hexosamine estimation. Hexose was estimated by the method of Niebes [30]. Briefly, the reaction mixture that contained 0.5 ml of aliquot/plasma, 0.5 ml of 5% phenol and 2.5 ml of concentrated H_2SO_4 was boiled for 20 min and absorbance was read at 490 nm.

Hexosamine was estimated by the method of Elson and Morgan [31], with slight modifications by Niebes [30]. Briefly, the reaction mixture that contained 0.5 ml of plasma/1.0 ml aliquot and 2.5 ml of 3 mol/l HCl was boiled over 6 h and neutralized with 6 mol/l NaOH. To 0.8 ml of the neutralized sample, 0.6 ml of acetyl acetone reagent was added and boiled for 30 min. The mixture was treated with 2.0 ml of Ehrlich's reagent and absorbance was read at 540 nm. Sialic acid was determined by the method of Warren [32]. Briefly, 0.5 ml of aliquot/plasma was treated with 0.5 ml of water, 0.25 ml of periodic acid and incubated at 37°C for 30 min. To the reaction mixture, 0.2 ml of sodium meta-arsenate and 2.0 ml of thiobarbituric acid were added and heated for 6 min. Later, 5.0 ml of acidified butanol was added to the preparation and absorbance was read at 540 nm.

Fucose was estimated by the method of Winzler [33]. Briefly, 1.0 ml of precipitated GP from platelet membrane and 1.0 ml of processed plasma were dissolved in 1 ml of 0.1 mol/l NaOH. The preparation was placed in an ice-bath and 4.5 ml of cold H_2SO_4 was added and mixed well. The tubes were heated in a boiling water bath for 3 min, cooled, added with 0.1 ml of 3% cysteine and mixed immediately. The tubes were allowed to stand at room temperature for 60–90 min. The absorbance was measured at 396 and 430 nm and the difference in the absorbance values were used for the calculations.

Statistical analysis

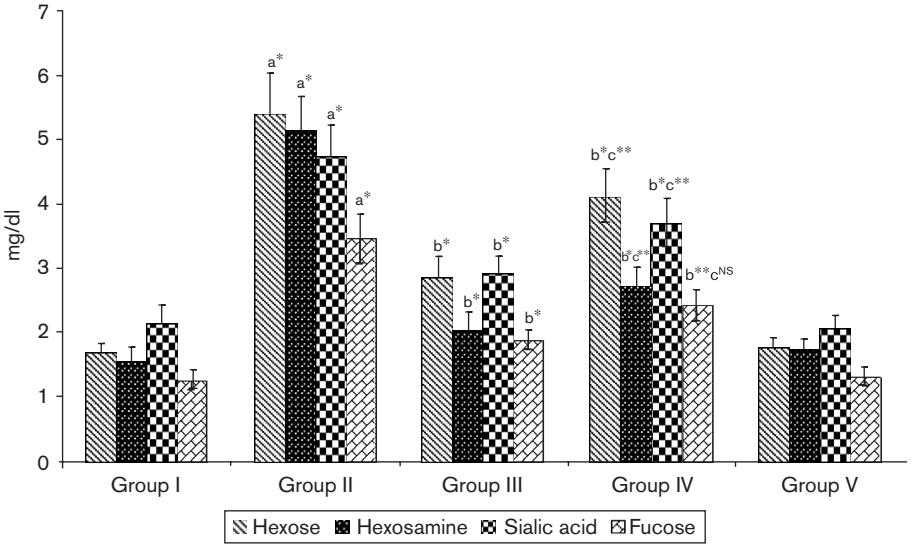
The data generated were calculated using analysis of variance. The group mean values were compared by the Duncan's multiple range test. P values of less than 0.05, 0.01 and 0.001 were considered statistically significant [34].

Results

The hexose, hexosamine, sialic acid and fucose levels estimated in the plasma of normal and experimental rats are shown in Fig. 1. A significant increase ($P < 0.001$) in the levels of plasma GPs was observed in the MNNG-induced gastric cancer rats when compared with group I animals. Administration of naringenin significantly ($P < 0.001$, < 0.01) reversed the plasma GPs of groups III and IV animals as compared with gastric cancer-induced group II animals. No significant change was seen among group V animals as compared with group I (normal) rats.

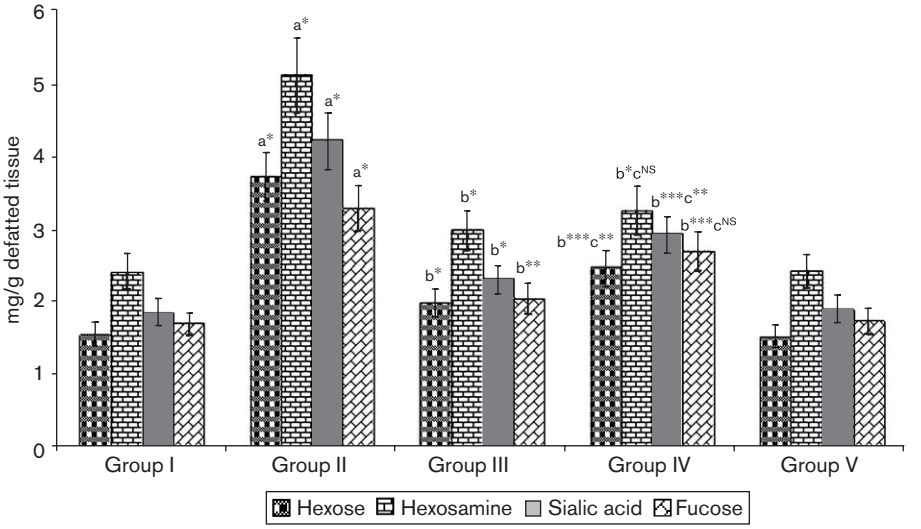
The liver hexose, hexosamine, sialic acid and fucose levels determined in the normal and experimental rats are shown in Fig. 2. Results revealed a significant increase ($P < 0.001$) in liver GP levels in the MNNG-induced gastric cancer animals as compared with control group I rats. Administration of naringenin significantly lowered ($P < 0.001$, < 0.01, < 0.05) the liver GPs of groups III and IV animals when compared with cancer-bearing

Fig. 1



Effect of naringenin on plasma glycoproteins in control and experimental rats. Groups (I–V) are control, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced cancer, naringenin pretreatment, naringenin posttreated and naringenin alone, respectively. The details are described in Materials and methods. Values are the mean ± SD of six rats in each group. a, As compared with group I; b, as compared with group II; c, as compared with group III. *$P<0.001$; **$P<0.01$; NS, not significant.

Fig. 2



Effect of naringenin on liver glycoproteins in control and experimental animals. Groups (I–V) are control, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced, naringenin pretreatment, naringenin posttreated and naringenin alone, respectively. Values are the mean ± SD of six rats in each group. Units, glycoprotein are expressed as mg/g of defatted tissue. a, As compared with group I; b, as compared with group II; c, as compared with group III. *$P<0.001$; **$P<0.01$; ***$P<0.05$. NS, not significant.

animals. No significant change was seen among group V animals as compared with group I rats.

Table 1 shows the level of stomach and kidney tissue GPs in normal and experimental rats. Among the cancer-induced rats, the level of hexose, hexosamine, sialic acid and fucose were significantly increased ($P < 0.001$) when compared with control rats. Administration of naringenin significantly reversed ($P < 0.001$, < 0.01, < 0.05) the stomach and kidney GPs of the gastric cancer-induced

Table 1 Effect of naringenin on levels of glycoproteins in stomach and kidney of control and experimental animals

Glycoprotein in stomach or kidney	Group I	Group II	Group III	Group IV	Group V
Stomach					
Hexose	0.33 ± 0.04	0.75 ± 0.08[a*]	0.46 ± 0.05[b*]	0.64 ± 0.05[b***,c*]	0.31 ± 0.04
Hexosamine	0.42 ± 0.03	0.88 ± 0.08[a*]	0.51 ± 0.04[b*]	0.66 ± 0.06[b*,c*]	0.43 ± 0.04
Sialic acid	0.27 ± 0.02	0.59 ± 0.05[a*]	0.35 ± 0.03[b*]	0.46 ± 0.04[b*,c*]	0.26 ± 0.03
Fucose	0.18 ± 0.02	0.47 ± 0.05[a*]	0.24 ± 0.03[b**]	0.36 ± 0.03[b**,cNS]	0.19 ± 0.02
Kidney					
Hexose	2.68 ± 0.28	4.24 ± 0.45[a*]	3.05 ± 0.33[b*]	3.86 ± 0.41[b**,c***]	2.71 ± 0.29
Hexosamine	3.16 ± 0.32	5.28 ± 0.55[a*]	3.92 ± 0.44[b***]	4.63 ± 0.49[b***,cNS]	3.19 ± 0.31
Sialic acid	1.93 ± 0.19	3.46 ± 0.35[a*]	2.33 ± 0.26[b**]	3.02 ± 0.33[b**,cNS]	1.95 ± 0.20
Fucose	1.78 ± 0.18	3.17 ± 0.33[a*]	2.11 ± 0.24[b*]	2.69 ± 0.31[b**,c***]	1.81 ± 0.19

Groups (I–V) are control, MNNG-induced, naringenin pretreatment, naringenin posttreated and naringenin alone, respectively. Values are the mean ± SD of six rats in each group. Units: glycoproteins are expressed as milligrams per gram of defatted tissue.
NS, not significant.
[a]As compared with group I.
[b]As compared with group II.
[c]As compared with group III.
*P < 0.001; **P < 0.01; ***P < 0.05.

rats as compared with MNNG-induced group II rats. Animals treated with naringenin alone (group V) did not show marked difference when compared with group I control animals.

Discussion

Increased levels of GPs have been shown to occur frequently in neoplasia [35]. The study results show that naringenin could possibly revert MNNG-induced gastric carcinogenesis in rats. So far, very few studies have reported the implication of GP with its biological functions. GPs, found on the surface of cells, are released into the bloodstream and other body fluids, and are essential for cell–cell communications [36–38]. The increase in circulating GPs may be because of increased activities of glycosidases and GP degradation. Similarly, other reports have also shown that GP components are altered in neoplasia [39,40]. The increased levels of hexose, hexosamine, fucose and sialic acid GPs observed among the MNNG-induced gastric cancer animals reversed to near normalcy after the administration of naringenin.

Recent studies from our laboratory showed an increase of cell proliferation in benzo(a)pyrene-induced lung cancer in mice could lead to neoplastic changes and it is decreased by piperine and other plant extract [41,42]. Another report has shown that elevation of GPs could be a valuable indicator of carcinogenesis [43] and therefore our study suggests that administration of naringenin could effectively reverse these changes.

Alteration of the expression of carbohydrate structures is frequently observed in tumour cells and also changes in O-linked and N-linked GPs are reported to occur in cancer cells. The impact of the tumour-related carbohydrate phenotypes on the clinical outcome of the cancer disease and the various ways in which carbohydrate structures can interact with different carbohydrate-detecting adhesion molecules, selectins and sialoadhesins are important factors in determining tumourigenesis [44]. Our results suggest that pretreatment could be better as compared with posttreatment, which could be attributed to naringenin-treatment changes of these types of molecules. GPs are being used frequently for diagnostic and prognostic purposes in squamous cell carcinoma [11].

On the basis of the available information concerning the increased level of fucose and hexosamine in MNNG-induced group II rats, administration of naringenin reduces the fucose and hexosamine in group III pretreatment than that of group IV rats. Although hexosamine branch groups may turn over independently of and more rapidly than other portions of the oligosaccharide, it would seem that the addition of a terminal fucose or sialic acid residue to an oligosaccharide chain confers a certain measure of metabolic stability on these oligosaccharides.

Sialic acid is widely distributed in mammals and occurs as a terminal component at the nonreducing end of the carbohydrate side chains of GPs and glycolipids. Researchers have documented that an increase in plasma sialic acid could be suggestive of cervical carcinoma [45]. Although the exact cause of increase in sialic acid levels in malignancy is unclear, a few theories have proposed certain correlates such as: alterations on the cell surface during malignant transformation [46] and tissue growth; provocating the liver to synthesize excessive GPs; increased glycosylation; shedding from the tumour cell surface; a constituent of a circulating tumour-associated antigen. Their implication in a variety of surface-related vital cell functions in tissues has been well documented. Sialic acid-rich glycoconjugates could also mask the surface of tumour cells by interfering with the host immune responses [47]. It also seems that sialic acid content is directly proportional to the magnitude of metastasis in tumour cells [48,49]. Sialic acid levels were

reversed in response to naringenin treatment [50]. Therefore, increased sialic acid concentration in cancerous animals is of considerable interest owing to its potential application as a diagnostic and prognostic marker of MNNG-induced gastric cancer.

Fucose, a component of glycocalyx of malignant cells, is incorporated to proteins in the α-1, 6 linkage to the proximal *N*-acetylglucosamine of the chitobiose core and in the α-1, 2 linkage on terminal galactose residues. An increased fucose metabolism could be associated with malignancy, which seems to affect protein turnover [51,52]. Our results support this concept. Furthermore, recent studies have also emphasized that excessive accumulation of fucose in the blood could result in fucosylation of haptoglobin in ovarian cancer [53]. Others have reported that the presence of fucose could decelerate the average turnover rate of gastric GPs [54]. This could be attributed to the fact that fucose is always located at a terminal position on the oligosaccharide chain and cannot shift more slowly than the hexosamine residues located at more internal positions. Although hexosamines might turn over independently of and more rapidly than other portions of the oligosaccharide, it seems that the addition of a terminal fucose or sialic acid residue to an oligosaccharide chain would provide metabolic stability to these oligosaccharides. The increase in GP ratio suggests an increased GP content in the gastric mucosa [55,56]. The decrease in protein levels of gastric juice is indicative of decreased leakage of plasma proteins into the gastric juice [57]. In this context, the action of naringenin, which protects the gastric mucosa, needs to be stressed. Recently, some polyphenolics have been identified to possess gastroprotective properties in rats. Others have focused on antiulcer activities of certain polyphenols [58], owing to strong antioxidant potentials and strong protein-binding abilities [59].

Naringenin could alter cell membrane GP synthesis kinetics and structure, presumed to be because of its potent reactive oxygen species-scavenging properties. This study has shown that naringenin was more effective in group III than group IV treated animals with regard to prevention correlates of stomach cancer. Therefore, we conclude that naringenin could effectively suppress malignant transformation by decreasing the degree of gastric cancer growth and controlling tissue proliferation.

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