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Altered membrane lipid dynamics and chemoprevention by non-steroidal anti inflammatory drugs during colon carcinogenesis

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Abstract

The present work focuses on the anti-neoplastic role of non steroidal anti-inflammatory drugs (NSAIDs) in modulating the biophysical parameters of the colonic membranes in 1,2-dimethylhydrazine dihydrochloride (DMH) induced carcinogenesis. The steady-state fluorescence polarization technique was applied to assess membrane fluidity, membrane polarity and lipid phase states. The decline in cholesterol content, biosynthesis and cholesterol: phospholipids ratio with DMH treatment indicates more fluidity associated with carcinogenesis. The DMH group had shown lower order parameter indicating more fluidity whereas NSAIDs resulted in increasing the membrane lipid order. The converging effects of these changes were more in membrane phase separations and membrane phase state. In DMH treatment membrane shows lesser phase separation or high polarity, and more liquid crystalline state while for NSAID groups membranes have higher phase separations or low polarity, and more of the gel phase. Further, NSAIDs induced anti-proliferative effects were evidently observed by apoptosis in the colonocytes by using acridine orange-ethidium bromide fluorescent staining and Terminal de-oxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The results suggest that NSAIDs induced alteration in the membrane biophysical parameters may be an important initiating event for the chemopreventive action.

Key words: Lipid phase separations, Membrane polarity, Membrane fluidity, Apoptosis, NSAIDs.

ALTERACIÓN DE LA DINÁMICA DE LOS LÍPIDOS DE MEMBRANA Y QUIMIOPREVENCIÓN MEDIANTE LOS FÁRMACOS ANTIINFLAMATORIOS NO ESTEROIDEOS EN LA CARCINOGÉNESIS DE COLON

Resumen

Este trabajo se centra en el papel antineoplásico de los fármacos antiinflamatorios no esteroides (AINE) en la modulación de los parámetros biofísicos de las membranas colónicas en la carcinogénesis inducida por 1,2-dihidrocloruro de dimetilhidracina (DMH). Se aplicó la técnica de polarización de la fluorescencia en estado de equilibrio para evaluar la fluididad de la membrana, su polaridad y los estados de fase lipídicos. El declive del contenido de colesterol, la biosíntesis y el cociente colesterol: fosfolípidos con el tratamiento con DMH indica más fluididad asociada con la carcinogénesis. El grupo DMH mostraba un parámetro de menor orden, lo que indica más fluididad, mientras que los AINE produjeron un aumento del orden de lípidos de membrana. Los efectos convergentes de estos cambios fueron más notables en las separaciones de la fase de membrana y en el estado de fase de membrana. Con el tratamiento con DMH, la membrana muestra menor separación de fase o polaridad elevada, y un estado cristalino más líquido o polaridad elevada mientras que los grupos de AINE tienen mayores separaciones de membrana o polaridad baja y más fase en estado gel. Además, los efectos antiproliferativos inducidos por los AINE se observaron de forma evidente utilizando tinción fluorescente con naranja de acridina-bromuro de etidio y el ensayo de marcado final de la dUTP transferase desoxinucleotid terminal (TUNEL). Los resultados sugieren que los AINE inducirían una alteración de los parámetros biofísicos de la membrana, lo cual podría ser un acontecimiento inicial importante para la acción quimiopreventiva.

Palabras clave: Separaciones en fase lípidica, Polaridad de la membrana, Fluidez de la membrana, Apoptosis, AINE.
Introduction

In recent years, modifications of physical characteristics of the membrane lipid bilayer, such as the fluidity changes along with membrane phase state and lipid phase separations, have proved important to clarify biological mechanisms and elucidate a variety of pathological conditions, such as, chronic inflammatory conditions and carcinogenesis. Many pathological cases including cancer are manifested in abnormal lipid composition of cell membranes which are perhaps the least understood phenomena. Indeed, a relationship has been suggested between the ability of the drugs to change membrane fluidity and their therapeutic action. Non steroidal anti-inflammatory drugs (NSAIDs) are the most important agents used in the treatment of inflammation, pain and fever and currently being seen as one of the best therapeutics for colon cancer chemoprevention. Like other drugs, the therapeutic effects of NSAIDs are strongly influenced by their relative lipid affinity which can have several consequences, including an induced perturbation of the physicochemical state of the membrane. Earlier, we have presented the evidence of pathogenesis of colon cancer and chemoprevention with NSAIDs along with the relationship of membrane lipid composition, fluidity and antioxidant defense in the morphogenesis of chemically induced neoplasm of rat intestine. Also it is shown that during colon carcinogenesis the NSAIDs induced chemoprevention is mediated through the alteration of membrane bound sodium-proton exchanger and low intracellular pH, followed by mitochondrial dysfunctioning by Ca²⁺-ATPase inhibition and an enhanced intracellular Ca²⁺ flux which may further lead to activate a strong apoptotic signal.

In the present study the chemopreventive actions of three NSAIDs were analyzed for lipid composition and functions of the colonic plasma membranes as a possible initiating event for the anti-proliferative role. The three NSAIDs chosen were Aspirin, Celecoxib and Etoricoxib for their inhibitory action against the two isoforms of Cyclooxygenase (COX), COX-1 and COX-2, with different selectivity. Aspirin, a non selective COX inhibitor, is a benchmark NSAID and represents the class of traditional-NSAIDs (tNSAIDs) whereas Celecoxib and Etoricoxib are from "coxib" family representing the second-generation NSAIDs. Although, both the coxibs selectively inhibit COX-2, Etoricoxib has the highest selectivity ratio of any coxib for inhibition of COX-2 relative to COX-1. These newly developed coxibs are considered to have tremendous potential in cancer chemoprevention as demonstrated in epidemiological studies, animal experimentation, cell culture and in clinical studies. Although evidences strongly suggest that NSAIDs induce their anti-proliferative effect by triggering programmed cell death (apoptosis) which primarily follows through the inhibition of the COX-2 pathway, the molecular mechanism of such inhibition is far from clear. Therefore, in order to have an understanding of the outcomes of altered membrane lipid components and phase changes, the status of apoptosis in the colonocytes was looked for the possible pathway of cancer preventing mechanism by the NSAIDs.

Material and methods

Animal care and drug treatment

Male Sprague-Dawley rats weighing 140-150 g were procured from the Central Animal House of Panjab University and housed in polypropylene cages under hygienic conditions, ambient light and temperature (18-22°C). They were fed with pellet diet and drinking water ad libitum and assorted into the following groups:

- Group 1- Control (vehicle treated);
- Group 2- DMH treated;
- Group 3- DMH + Aspirin treated;
- Group 4- DMH + Celecoxib treated; and
- Group 5- DMH + Etoricoxib treated.

Animals in the groups 2 to 5 were given DMH weekly at a dose of 30 mg/kg body weight subcutaneously (s.c.) for 6 weeks. In addition, the animals in group 3 to 5 received daily dose of the respective NSAIDs (viz., Aspirin- 60 mg, Celecoxib- 6 mg and Etoricoxib- 0.6 mg) orally per kg body weight. Animals were acclimatized for a period of one week prior to the experimental work.

1,2-dimethylhydrazine dihydrochloride (DMH) (Sigma, USA) was prepared fresh immediately before injection in 1 mM EDTA-saline, pH being adjusted to 7.0 using 100 mM NaOH. Aspirin, Celecoxib and Etoricoxib were generous gift from Ranbaxy Research Lab (Gurgaon, India). Their reported anti-inflammatory doses were prepared fresh every day in 0.5% sodium carboxymethyl cellulose (CMC). The vehicle treated animals were administered with 0.5% CMC everyday per oral and 1mM EDTA saline solution once weekly by s.c. injection. The bodyweight of the rats was recorded weekly till the termination of the experiments. They were sacrificed in overnight fasted conditions 6 weeks after beginning of the study by keeping them under an overdose of ether anesthesia and sacrificed around 8 AM to avoid diurnal variation in the parameters. Animal procedures were approved by Panjab University Ethical Committee on Experimental Animals for Biomedical Research. The guidelines were prepared according to the principles of animal care laid down by the National Institute of Health, USA (NIH publication No. 23-85, revised in 1985).

Isolation of colonic brush border membrane (BBM)

The BBM of rat colon was isolated using the method of Brassitus and Keresztes and had been variously described
in earlier publications. The final membrane so obtained was analyzed for the membrane specific enzyme, cysteine sensitive alkaline phosphatase.

Isolation of colonocytes

Colonocytes were obtained from the freshly dissected colons by the method of Mouille et al. The integrity of the colonocytes was assessed by the ability of the cells to exclude vital dyes. The number of colonocytes re-suspended in DMEM buffered with MOPS (pH 7.5, 25 mM) were counted on a hemocytometer and it was determined that the isolation procedure led to the recovery of the viable colonocytes, to the extent of at least 97%. In order to assess the efficiency of colonic epithelial cell retrieval, pieces of mucosa were fixed in 10% formalin saline both before and after cell separation. The tissue was processed for paraffin embedding and then the sections stained with hematoxylin and eosin.

Quantitative analysis of Lipids

Extraction of Lipids from the colonic mucosa was done by the method of Folch et al and as described variously earlier. The lipid extracts obtained were stored at -20°C and used as such for various lipid estimations. The pooled upper aqueous phase (also called the Folch upper phase) was used for ganglioside estimation. Total lipids were estimated by the method of Frenge and Dunn, phospholipid by the method of Ames, cholesterol by the method of Zlatkis et al and ganglioside-sialic acid by the method of Warren, respectively.

Dynamics of lipid metabolism

14C incorporation of Palmitic acid-1-C-14 (Bhabha Atomic Research Centre, Mumbai, India) in the membrane lipids was studied by incubating the colonic tissues in the radioactive substrate and then isolating the lipid by thin layer chromatography and quantitating by liquid scintillation spectrometry following the method of Butler and as described earlier.

Liposome preparation

Liposomes were prepared from the extracted lipids by the method described earlier. A known volume of lipid extract (in chloroform: methanol) was evaporated to dryness at room temp and suspended in sodium maleate buffer to a final conc. of approx 2.5 mg/ml. The mixture was sonicated for 1 min with brief pause in between, 2-3 times using a probe type sonicator (Sonics, USA) in an ice cold environment. The suspensions were centrifuged for 10 min at 50,000 g in a Beckman Coulter ultracentrifuge. The supernatant liposomes were tested with the various fluorescent probes.

Fatty acid analysis

Fatty acid methyl esters were prepared as described earlier and quantitated in a gas liquid chromatography (GLC) set up. Appropriate standards were also run simultaneously. Conditions for GLC were such that the injection temp was set at 230°C, column temp at 220°C, detector temp at 240°C, and the carrier gas was Nitrogen. Flame ionizing detector was used and the column was of FFAP from Machery Nagel GmbH, Duren, Germany. Retention times of different fatty acids were recorded and the results expressed in term of weight %.

Evaluation of lateral phase separation by N-NBD-PE fluorescence quenching

Membrane lipid phase separations were monitored in the liposomes, BBM and colonocytes by the method as described by Hoekstra using the self-quenching of N-NBD-PE fluorescence. The method is based on the self quenching of the NBD fluorophore that occurs when the local concentration of NBD lipid in the bilayer increases during the segregation of the membrane lipids into the discrete domains in the plane of the bilayer. 2ml vol containing approx 150 µg of BBM/O.25 mg liposomes or colonocytes (2 x 10^5 cells) were labeled with N-NBD-PE (final concentration 0.6 µM) (Sigma, USA) by incubating the tubes in dark for 15 min at 37°C. The samples of colonocytes and BBM were loaded with N-NBD-PE using the same buffer as was used for their original isolation procedure while in case of liposomes, the small unilamellar vesicles were generated in 0.1 M NaCl/0.01 M Hepes, pH 7.4, by ultrasonication (Sonics, USA) in cold conditions and ultracentrifugation (at 150,000 g for 1 h). Fluorescence assay was done by measuring the fluorescence intensities at λem = 475 nm and λex = 530 nm before and after the solubilization of vesicles or cells as described by Nicholas & Pagano. BBM vesicles/liposomes or colonocytes were solubilized by the addition of Triton X-100 (100 µl). The percentage of quenched fluorescence (% Q) was calculated from the relative fluorescence of unquenched N-NBD-PE (F0) in the presence of detergent and quenched molecules (FQ) in vesicles by the relation:

\[ Q = \frac{|F_0 - F_Q|}{F_0} \times 100 \]

Evaluation of membrane phase state

Membrane phase determination was done to evaluate the membrane polarity in terms of generalized polariz-
tio (GP) using fluorescence probe, Laurdan (6-dodecanoyl-2-dimethylamino-naphthalene) following the method as described by Ambrosini et al. Laurdan is particularly sensitive to the phase state of the membrane. The basis of its spectral sensitivities lies in its ability to sense the polarity change of the membrane and is therefore, a powerful technique to detect physico-chemical modifications. The environment-sensitive spectroscopic properties of Laurdan have been comprehensively described by a parameter termed generalized polarization (GP), originally introduced by Gratton and co-workers. GP values from excitation and emission spectra are characteristic of the membrane phase state. In order to quantify the GP parameter in biological membranes the relative values of exGP and emGP, (measured on exGP at 340 and 400 nm) and emGP and emGP, (measured on emGP at 440 and 490 nm) are considered important.

Calculation of generalized polarization (GP) parameter

150 μg of BBM/0.25 mg liposomes/2 x 10^5 cell population of colonocytes were equilibrated for 20 min at 37°C in the dark to be labeled with Laurdan (stock prepared in methanol) (Sigma, USA) at 1.4 μM final conc. The buffers for BBM, liposomes and colonocytes were kept the same as that of their original preparations for labeling the probe. Steady-state Laurdan excitation spectra were obtained in the range of 300 to 420 nm, using both 440 and 490 emission wavelengths. Similarly, Laurdan emission spectra were recorded in the range from 420 to 550 nm, using both 340 and 410 nm excitation wavelengths. Blank spectra were obtained with unlabelled samples and subtracted from the spectra of the labeled samples. Readings were made at a fixed temp of 37°C for all the three sample systems. From the spectroscopic data, Laurdan excitation generalized polarization (exGP) spectra were derived by calculating the GP value for each excitation wavelength using Eq. (1): exGP = (I_440 - I_490)/(I_440 + I_490) where I_440 and I_490 are the intensities measured at each excitation wavelength (from 320 to 420 nm), on the fluorescence excitation spectra obtained by fixed emission wavelength of 440 and 490 nm, respectively. Laurdan emission generalized polarization (emGP) spectra were derived by calculating the GP value for each emission wavelength using Eq. (2): emGP = (I_340 - I_490)/(I_340 + I_490) where I_340 and I_490 are the intensities measured at each emission wavelength, from 430 to 520 nm. These values are obtained by the fluorescence emission spectra recordings using fixed excitation wavelength of 410 and 340 nm, respectively. The choice of 410, 340, 440 and 490 nm for GP calculation was based on the characteristic excitation and emission wavelengths of pure gel and liquid-crystalline lipid phases, according to Parasassi et al.

Fluidity and order parameter assay using DPH

The fluorescent probe, 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) (Sigma, USA) was used in the fluidity studies (rotational diffusion). A stock solution of 1 mM probe in tetrahydrofuran (THF) was prepared and stored in dark colored bottle at room temp. A small vol (10 μl) of DPH solution in THF was injected with rapid stirring into 1,000 vol of Tris/HCl buffer (10 ml) at room temp. The suspension was stirred for at least 2 h after which little or no odor of THF was detected and the suspension showed negligible fluorescence. The BBM were incubated in 3 ml of the above suspension containing 3 μl DPH for 2-4 hr at 37°C. Thereafter, estimations of fluorescence intensity (I), fluorescence polarization (P), and fluorescence anisotropy (r) were made with an excitation wavelength of 365 nm and emission wavelength of 430 nm using a Perkin Elmer Luminescence Spectrometer: LS-55 (Beaconsfield, United Kingdom). Order parameter (S) was calculated using the formula, S = [(4r - 3)/(3r - 1)]/r [25] taking r value for DPH as 0.362 and fluidity was measured in term of reciprocal of fluorescence polarization (1/P) values and as described by us earlier.

Assessment of apoptosis in colonocytes

Acridine orange-ethidium bromide co-staining

Acridine orange is a fluorescent agent, which can intercalate (slip in) between base pairs (bp) in the central stack of DNA helix. This staining procedure was performed according to the details provided by Baker et al. Briefly, the cells were suspended in PBS (pH 7.4, HiMedia, Mumbai) containing acridine orange (1 μg/ml) and RNaseA (Sigma, USA) as well as ethidium bromide (Sigma, USA) in the same concentration. The cells were washed and examined under fluorescence microscope (x400) (Axioplan, Zeiss, Germany). For quantification of apoptotic cells, a total of 100 cells from four different slides were observed and percentages of apoptotic cells calculated for the individual animal.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL is a specific method for detecting DNA fragmentation (damage) that results from the apoptotic signaling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. TUNEL assay was performed according to the protocol provided by Trevigen (using TACS-XL® in situ apoptosis detection kit from Trevigen (USA) which is based on the incorporation of bromodeoxyuridine (BrdU) at the 3' OH ends of the DNA frag-
**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TL (mg/g tissue)</th>
<th>Ch (mmole/g tissue)</th>
<th>PL (mmole/g tissue)</th>
<th>Ch/PL (mole/mole)</th>
<th>GSA (nmole/g tissue wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.93 ± 1.55</td>
<td>11.724 ± 0.171</td>
<td>2.603 ± 0.049</td>
<td>4.504 ± 0.078</td>
<td>3.570 ± 0.108</td>
</tr>
<tr>
<td>DMH</td>
<td>15.17 ± 0.45</td>
<td>7.068 ± 0.564</td>
<td>6.721 ± 0.392</td>
<td>1.057 ± 0.145</td>
<td>5.870 ± 0.206</td>
</tr>
<tr>
<td>DMH + Aspirin</td>
<td>18.58 ± 2.73</td>
<td>7.529 ± 0.395</td>
<td>4.619 ± 0.131</td>
<td>1.629 ± 0.060</td>
<td>5.627 ± 0.107</td>
</tr>
<tr>
<td>DMH + Celecoxib</td>
<td>19.33 ± 2.14</td>
<td>8.010 ± 0.517</td>
<td>3.747 ± 0.199</td>
<td>2.172 ± 0.254</td>
<td>5.256 ± 0.090</td>
</tr>
<tr>
<td>DMH + Etoricoxib</td>
<td>15.87 ± 1.42</td>
<td>12.701 ± 1.294</td>
<td>4.091 ± 0.131</td>
<td>3.100 ± 0.243</td>
<td>5.287 ± 0.152</td>
</tr>
</tbody>
</table>

The values are mean ± S.D. of 8 animals. Significantly different from control at *p* ≤ 0.001 and DMH at *p* ≤ 0.01, *p* ≤ 0.001 by one way ANOVA.

**Statistical analysis**

Statistical examination of the data was performed by the analysis of variances (one way ANOVA) following Post-Hoc test using the least significant difference (LSD) method with the help of SPSS v 14 computer software (SPSS Inc. Chicago, IL, USA).

**Results**

**Quantitative analysis of lipids**

Table 1 depicts the profiles of the total lipid (TL), cholesterol (Ch), phospholipid (PL), cholesterol: phospholipid ratio (Ch/PL) and ganglioside sialic acid (GSA) content present in the colonic mucosa. It showed total lipid and cholesterol value to decrease in case of DMH treatment which was recovered in the NSAIDs groups. The phospholipid content was found to be increased with DMH treatment and similarly corrected in the NSAIDs groups. Among the NSAIDs tested, the values in the Etoricoxib + DMH reached close to the control. Chol:PL ratio was found to be reduced remarkably with the DMH administration, however, in comparison to DMH group a significant improvement was observed with the NSAIDs treatment and the maximum recovery of the Chol:PL ratio was observed in DMH + Etoricoxib. DMH group has also resulted in an increase in the PL, which was lowered in the DMH + NSAID groups. Levels of GSA were found to be elevated in the DMH group as compared to the control whereas, DMH + NSAIDs treatment caused the substantial decrease in comparison to the DMH only group.

**Dynamics of lipid metabolism**

Results of membrane lipid and phospholipids biosynthesis studies using 1-14C-labelled palmitic acid incorporation are shown in the table II. A level of the radioactivity incorporated into the cholesterol was found to be decreased with DMH as compared to the control. Co-administration of Aspirin and Celecoxib proved helpful in recovering the cholesterol biosynthesis in comparison to DMH while DMH + Etoricoxib group recorded the highest increase in the same. Free fatty acids (FFA) were observed to be unaltered in the DMH while in NSAIDs treated groups, FFA was significantly low in comparison to DMH as well as the control group. Triglycerides (TG) registered a significant increase in DMH group as compared to the controls while NSAIDs treatment reduced its biosynthesis when compared to the DMH group. The lower level of triglycerides was recorded in the DMH + Celecoxib and DMH + Etoricoxib groups. Similarly, the fraction corresponding to the monoglycerides + diglycerides was observed to be lower in the DMH + NSAID groups when compared to the DMH alone. The DMH alone registered a slight increase over the control. Cholesterol ester was observed to be unaltered in DMH group as compared to the control while higher levels were recorded in the DMH + NSAID groups. S, PC and PE were observed to be enhanced in the DMH group whereas with NSAIDs these fractions recorded lower concentrations in comparison to DMH group. Fraction corresponding to PI + PS content showed an increment in the DMH + NSAIDs groups with respect to the DMH.

**Fatty acid analysis**

Table III shows the quantitative values (wt %) as derived from the elution profile of various fractions (wt %) of fatty acid methyl ester derivatives. Carbon 16 fatty acids, palmitic acid (16:0) and palmitoleic acid (16:1) contents were observed to decrease in DMH group while NSAID showed an increase. Increment of the carbon 18 fatty acids, stearic acid (18:0) increased in DMH group while NSAID caused the decrease. Higher unsaturated fatty acids such as the linoleic acid (18:2) and arachidonic acid (20:4) showed an increase in the DMH group as expected, which declined subsequently in the NSAIDs groups. Etoricoxib in particular. Overall results
Table II

(a) Effect of different treatments on the biosynthesis of membrane neutral lipid fractions: free fatty acids, cholesterol, triglycerides, mono- + di-glycerides and cholesterol ester as measured by \(^{14}C\)-labelled palmitic acid incorporation.

(b) Effect of different treatments on the biosynthesis of membrane phospholipid fractions: sphingomyelin (S), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) as measured by \(^{14}C\)-labelled palmitic acid incorporation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Palmitic acid</th>
<th>Stearic acid</th>
<th>Free Mono-Glycerides</th>
<th>Palmitic acid</th>
<th>Stearic acid</th>
<th>Palmitic acid</th>
<th>Stearic acid</th>
<th>Palmitic acid</th>
<th>Stearic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>60.2 ±3.5</td>
<td>49.8 ±3.3</td>
<td>60.2 ±3.5</td>
<td>49.8 ±3.3</td>
<td>60.2 ±3.5</td>
<td>49.8 ±3.3</td>
<td>60.2 ±3.5</td>
<td>49.8 ±3.3</td>
<td>60.2 ±3.5</td>
</tr>
<tr>
<td>DMH</td>
<td>45.6 ±2.1</td>
<td>54.4 ±2.9</td>
<td>45.6 ±2.1</td>
<td>54.4 ±2.9</td>
<td>45.6 ±2.1</td>
<td>54.4 ±2.9</td>
<td>45.6 ±2.1</td>
<td>54.4 ±2.9</td>
<td>45.6 ±2.1</td>
</tr>
<tr>
<td>DMH + Aspirin</td>
<td>31.4 ±1.5</td>
<td>68.6 ±1.5</td>
<td>31.4 ±1.5</td>
<td>68.6 ±1.5</td>
<td>31.4 ±1.5</td>
<td>68.6 ±1.5</td>
<td>31.4 ±1.5</td>
<td>68.6 ±1.5</td>
<td>31.4 ±1.5</td>
</tr>
<tr>
<td>DMH + Celecoxib</td>
<td>16.6 ±0.2</td>
<td>83.4 ±0.6</td>
<td>16.6 ±0.2</td>
<td>83.4 ±0.6</td>
<td>16.6 ±0.2</td>
<td>83.4 ±0.6</td>
<td>16.6 ±0.2</td>
<td>83.4 ±0.6</td>
<td>16.6 ±0.2</td>
</tr>
<tr>
<td>DMH + Etoricoxib</td>
<td>11.2 ±0.1</td>
<td>90.8 ±1.0</td>
<td>11.2 ±0.1</td>
<td>90.8 ±1.0</td>
<td>11.2 ±0.1</td>
<td>90.8 ±1.0</td>
<td>11.2 ±0.1</td>
<td>90.8 ±1.0</td>
<td>11.2 ±0.1</td>
</tr>
</tbody>
</table>

The values are mean ± S.D. of 8 animals. Significantly different from control at \(p < 0.05\), \(p < 0.01\) and DMH at \(p < 0.05\), \(p < 0.001\) by one way ANOVA.

Table III

Quantitative analysis of fatty acid (weight %) as obtained from the elution profiles from GLC of fatty acid methyl ester derivatives

<table>
<thead>
<tr>
<th>Acid</th>
<th>Control</th>
<th>DMH</th>
<th>DMH + Aspirin</th>
<th>DMH + Celecoxib</th>
<th>DMH + Etoricoxib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid</td>
<td>2.0 ±0.05</td>
<td>1.8 ±0.02</td>
<td>1.6 ±0.02</td>
<td>1.7 ±0.01</td>
<td>1.7 ±0.01</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>31.1 ±0.03</td>
<td>28.8 ±0.04</td>
<td>29.5 ±0.02</td>
<td>32.1 ±0.08</td>
<td>33.3 ±0.50</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>7.2 ±0.28</td>
<td>4.4 ±0.16</td>
<td>7.5 ±0.50</td>
<td>8.1 ±0.24</td>
<td>5.70 ±0.67</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>7.3 ±0.94</td>
<td>9.2 ±0.11</td>
<td>7.3 ±0.99</td>
<td>7.3 ±0.92</td>
<td>8.1 ±0.51</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>37.2 ±1.9</td>
<td>39.1 ±2.3</td>
<td>39.0 ±1.6</td>
<td>41.8 ±1.38</td>
<td>37.9 ±1.46</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>11.9 ±0.51</td>
<td>12.9 ±0.32</td>
<td>12.5 ±0.64</td>
<td>8.3 ±0.46</td>
<td>12.1 ±0.48</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>2.3 ±0.07</td>
<td>3.7 ±0.30</td>
<td>2.6 ±0.29</td>
<td>1.1 ±0.88</td>
<td>1.5 ±0.05</td>
</tr>
</tbody>
</table>

The values are mean ± S.D. of 8 animals. Significantly different from control at \(p < 0.05\), \(p < 0.01\) and DMH at \(p < 0.01\), \(p < 0.001\) by one way ANOVA.

Table IV

Saturation and unsaturation calculated from the quantitative data obtained from the elution profiles of fatty acid methyl ester derivatives from GLC

<table>
<thead>
<tr>
<th>Acid</th>
<th>Saturation (%)</th>
<th>Unsaturation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.4 ±0.12</td>
<td>58.6 ±2.78</td>
</tr>
<tr>
<td>DMH</td>
<td>39.8 ±0.19</td>
<td>60.1 ±2.26</td>
</tr>
<tr>
<td>DMH + Aspirin</td>
<td>38.4 ±0.11</td>
<td>61.6 ±2.58</td>
</tr>
<tr>
<td>DMH + Celecoxib</td>
<td>40.7 ±1.02</td>
<td>59.3 ±2.51</td>
</tr>
<tr>
<td>DMH + Etoricoxib</td>
<td>42.8 ±10.3</td>
<td>57.2 ±2.07</td>
</tr>
</tbody>
</table>

The values are mean ± S.D. of 8 animals. Significantly different from control at \(p < 0.05\) and DMH at \(p < 0.001\) by one way ANOVA.

Table V

Percentage of arachidonic acid (20:4) and palmitic acid (16:0) present in the lipids extract calculated from the quantitative data (wt%) obtained from the elution profiles of fatty acid methyl ester derivatives from GLC

<table>
<thead>
<tr>
<th>Acid</th>
<th>Control</th>
<th>DMH</th>
<th>DMH + Aspirin</th>
<th>DMH + Celecoxib</th>
<th>DMH + Etoricoxib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid</td>
<td>100 ±3.47</td>
<td>160.86 ±5.65</td>
<td>113.04 ±2.17</td>
<td>47.83 ±3.46</td>
<td>65.22 ±3.04</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>100 ±0.12</td>
<td>89.72 ±0.12</td>
<td>98.13 ±0.68</td>
<td>100 ±0.24</td>
<td>102.80 ±1.58</td>
</tr>
</tbody>
</table>

The values are mean ± S.D. of 8 animals. Significantly different from control at \(p < 0.05\), \(p < 0.001\) and DMH at \(p < 0.001\) by one way ANOVA.
Evaluation of lateral phase separation by N-NBD-PE fluorescence quenching

The effects of DMH and DMH + NSAIDs treatment on the physical state of BBM vesicles, liposomes and colonocyte membranes on the NBD fluorescence are shown in the figure 1. Results showed a decrease in % Q values in all the membrane systems for DMH group when compared with the control, which was in general rising in the DMH + NSAIDs. The effect was most prominent in case of the Etoricoxib group.

Evaluation of membrane phase state

Laurdan exGP and emGP spectra were measured from 320 to 400 nm and from 440 to 490 nm, respectively, to characterize the lipid phase of three different membrane bilayer systems, viz, liposomes, BBM and colonocytes. In order to quantify the slope changes, the relative values of exGP340 were measured on exGP spectra (at 340 nm) (fig. 2a) and emGP490 measured on emGP spectra (at 490 pm) (fig. 2b). The exGP340 value was found to be decreased in DMH group for all the membrane systems, while a significant improvement was observed for the DMH + NSAIDs and in particular, DMH + Etoricoxib group. ExGP340 is an important parameter of lipid phase state and membrane polarity as this value was shown to be directly related to the increasing cholesterol content of a membrane.¹⁴

Fluidity and order parameter assay using DPH

Fluorescence polarization was expressed as the fluorescence anisotropy (r), and as the anisotropy parameter \( r/(r+1) \), which are probe dependent phenomena and reflect the overall motional freedom of the fluorescent molecule without distinguishing the specific mechanisms affecting its motions. The specific mechanisms that affect the motion include alteration of the rate of rotation of the probe owing to viscous drag of the environment, anisotropic rotations, and hindered motions owing to structural factors. Thus, within certain limitations, anisotropy and anisotropy parameters are accepted as proportional to the fluidity. The results were obtained according to the modified Perrin relationship as given by Eq. (3): \( r = r_0 + (r_0 - r) (T / T_e + T) \), where \( r_0 \) is the maximal limiting anisotropy, \( r_0 \) is the limiting hindered anisotropy, \( T \) is the correlation time and \( T_e \) is the mean lifetime of the excited state. It should be noted, however, that with the present instrument (Perkin Elmer LS 55), \( r \) values were obtained directly from the instrument and anisotropy parameter calculated using \( r_0 = 0.365 \). The static component of the membrane fluidity was assessed by an order parameter (S) according to Eq. (4): \( S = [(r_0 - r) / r_0]^{1/2} \).

Results of order parameter as calculated from the observed anisotropy values are given in figure 3a and show the lowest S value and therefore, increased state of lipid fluidity in the DMH group while with the NSAID treatments the situation was reversed with higher S values and lesser fluidity. Similarly, the fluidity changes as calculated from the inverse of polarization values \((1/P)\) show an enhanced state of fluidity in DMH group which was corrected in the NSAID groups (fig. 3b). The lipid fluidity was observed minimum in DMH + Etoricoxib group as also shown by maximum ordered state of lipids (higher S value).

Morphological evaluation of apoptosis in colonocytes

Apoptosis in the colonocytes was studied by assessing the nuclear morphology using DNA

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Apoptosis in the colonocytes was studied by assessing the nuclear morphology using DNA
binding dyes acridine orange-ethidium bromide co-
staining as well as by the TUNEL assay in the
paraffin sections of the colonic tissue. Using fluo-
rescent DNA binding dyes apoptotic cells were indi-
cated by the appearance of brightly labelled nuclei
(bright orange coloured). Figure 4a–e shows the
Fig. 4.—Morphological features of colonicocytes from different treatments showing the induction of apoptosis as visualized by acridine orange & ethidium bromide co-staining: (a) Control, (b) DMH, (c) DMH + aspirin, (d) DMH + celecoxib and (e) DMH + etoricoxib. Arrows indicate apoptotic cells.

RESULTS

Photomicrographs of the appearance of normal and apoptotic cells as observed in the various groups using fluorescence microscopy while figure 5a-e shows the results of TUNEL assay. To quantify the extent of apoptosis from these studies percentage of apoptotic colonicocytes (as observed using fluorescence microscopy) as well as apoptotic nuclei (as observed under light microscope using TUNEL assay) were calculated and presented in figure 6. Results from both the studies showed similar trends. It was found that only a minimum number of apoptotic cells were present in the DMH group while an increased percentage of apoptotic cells observed in the DMH + NSAIDs groups. Maximum occurrence of apoptotic cells were recorded in the DMH + Etoricoxib group.

Discussion

Cell membrane is an integral part of the alive cell and plays an essential role in life processes; it makes the cell an isolated system and determines its specific properties under a well controlled equilibria comprising of proteins, phospholipids, neutral lipids and fatty acids. A cancer transformation results in the appearance of a new cell line in the organism whose malignant activity is transmitted from one cell generation to another and is connected with the changes in the cell membranes. Cell proliferation and tumor growth occur due to an increased rate of de novo synthesis of its constituent components. Also, cancer transformations occur at the cost of cell’s immune capacities and changes in membrane composition would affect growth, interac-

Fig. 5.—TUNEL assay for detecting the apoptotic nuclei (dark brown) in the colonic segments from different treatment groups. (a) Control section showing the occurrence of moderate apoptosis, (b) section from DMH showing the negligible amount of apoptosis, (c) DMH + aspirin showing the moderate levels of apoptosis, (d) DMH + celecoxib and (e) DMH + etoricoxib showing the increased presence of apoptotic cells.
tion with other cells (immune system), and the function of proteins and other components in the membrane.

Alterations in the synthesis, uptake, and membrane content of cholesterol have been observed in a variety of experimental tumor models as well as in human neoplasms. Cholesterol content and Chol:PL ratio are important from a malignancy point of view as also indicated by many previous reports. In the present study the decrease in the cholesterol content and its synthesis was observed in the DMH group which was found to be associated with the decrease in Chol:PL ratio along with increased triglyceride (TG) biosynthesis. These changes were found to be reversed with NSAIDs treatments. It has been found that cancer cell membranes have low cholesterol content and low Chol:Protein ratio. Chol:PL ratio is also closely related to the membrane fluidity. Addition of cholesterol is known to cause a transition from gel to liquid-crystalline phase resulting in alteration in the nature and extent of phospholipid-protein interaction and a decrease in amplitude of motion of the chain axes and reduced fluidity of the system. Similarly, TG contents have also been investigated in previous works to be involved in colorectal cancer. A study of Meckown-Eysen has showed the elevated levels of TG in the serum to be indirectly involved with colorectal cancer. An increased risk of colorectal adenomas was found among men with the highest level of serum TG in a Japanese study, therefore, an increased synthesis for the TG as observed in the DMH group seems to be an important finding.

Further increments were also observed in the biosynthesis of major phospholipids studied i.e., S, PC and PE (except for PI + PS fractions) in the DMH group while lower values were observed in NSAID groups. An increment in biosynthesis of phospholipids may be required for cell proliferation, especially during carcinogenesis, in particular, PC biosynthesis has been implicated in the process, while an inhibition of PC biosynthesis in tumor cell lines has been associated with an increased event of apoptosis. The increase in PC values in DMH group therefore, indicates the more proliferative capability while a decreased synthesis indicates the inhibition of PC biosynthesis in NSAID groups which is consistent with the enhanced role of the NSAIDs in apoptosis. Further, in NSAIDs groups increased PI was noted. An increased production of PI was observed to be associated with disruption of membrane integrity of both mitochondria and endoplasmic reticulum. Agents, such as etoposide, camptothecin, farnesol, and chelerythrine, and ether lipids which are known inducers of apoptosis have also been shown to inhibit PC biosynthesis, although the effects of these agents on PI biosynthesis have not been described.

Gangliosides are important membrane constituents that may influence growth and cell-to-cell interactions and therefore, play a vital role in the development of malignancy. Thus in the present study ganglioside was assayed in the lipids derived from the colonic mucosa to look into any possible NSAIDs mediated regression in colon carcinogenesis. The present results
showed an elevated level of gangliosides in the colon of DMH treated animals whereas significant regression was observed in the coxib treated groups. Previously, Lu et al. have shown that ganglioside contents in tumor tissues as well as the plasma sialic acid levels to be significantly increased in experimental tumorogenesis. Earlier studies have also demonstrated the alterations in the levels of ganglioside sialic acid to be associated with the process of carcinogenesis in various tissues.

Cancer causation is a process that require a constant supply of fatty acids from the organism’s metabolic apparatus and it is well established that lipid composition of the immune and tumor cell membranes are influenced by the fatty acid constituents. Fatty acids, for example: palmitoleic 16:1, oleic 18:1, linoleic 18:2, along with stearic 18:0, palmitic 16:0, myristic 14:0 and arachidonic acid 20:4 are known to potentially influence these interactions.

Fatty acids like arachidonic acid influences the synthesis of eicosanoids (prostaglandins, leukotrienes and thromboxanes) and in addition to their role in the regulation of immune and inflammatory responses, eicosanoids may also be needed to sustain growth of tumor cells. Palmitic acid on the other hand has been reported to have a key role in the mitochondrial membrane potential and cytochrome c release, thereby, inducing apoptosis in the cells. In view of this, lipids extracted from the colonic mucosa were analyzed for fatty acid composition including most importantly AA and palmitic acid. An increased level of AA was found in the colonic tissues obtained from the DMH group as compared with the control or NSAID treated groups. Previously, Nicholson et al. showed consistently high values of cell membrane AA in an experimental colorectal tumor tissue. A recent study and earlier clinical reports on the colorectal tissues of human cancer have also showed an increase in AA.

These studies contributed to the idea that the increase in AA is a feature of the rapidly growing cells. On the other hand, the increased level of palmitate in NSAID groups thus signifies their role as inducers of apoptosis. Induction of apoptosis by palmitate has also been observed in other cell types, including cardiomyocytes, hematopoietic cells, pancreatic β-cells, and astrocytes, but for most of these cell types, the mechanism by which palmitate induces the cell death remains elusive. It has been proposed that excess palmitate could induce cell death through increased intracellular concentration of ceramide, a metabolite exclusively produced from the saturated FFAs. Alternatively, other workers have suggested that apoptosis induced by palmitate could occur through the generation of the reactive oxygen species (ROS). In the present studies, apoptosis was evidently observed to occur in the NSAID groups as observed through fluorescent staining of colonocytes and TUNEL assay. These results were found to be in agreement to those of Liu et al. and Kim et al. and Xu et al., where apoptosis was found to be induced after NSAIDs administration. These observations taken together therefore strongly indicate the role of palmitate in preventing the DMH induced carcinogenesis possibly through apoptosis.

The segregation of membrane lipids in domains, called lateral phase separation assumes importance due to their regulatory role in cellular functions such as membrane sorting and signal transduction. Fluorescent lipid probes have been proved very useful due to their ability to monitor lipid molecules by a variety of physicochemical approaches at increasing spatiotemporal resolution. A widely used extrinsic fluorophore in the studies of membrane is the NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) group which is very weakly fluorescent in water and upon transfer to a hydrophobic medium, fluoresces brightly in the visible range and also exhibits a high degree of environmental sensitivity. Various fluorescent analogues of native lipids labeled with NBD have been used to study a variety of biological processes such as cell growth and proliferation, apoptosis and signal transduction. Originally, Hoekstra presented a method for monitoring lipid phase separation by using small amount of fluorescent phospholipids (NBD labelled) analogues incorporated into the vesicle bilayer, which become self quenched during the process of phase separation. Separation of lipid phase leads to an increase in local concentrations of the NBD-lipid in the bilayer, and hence to self quenching of NBD fluorescence. The rate and extent of NBD self quenching provides the measure of the rate and extent of phase separation in membrane bilayers.

The increase in %Q was found to be a larger extent in case of DMH + Etoricoxib group, both in comparison to control as well as DMH group. This indicates that under present experimental conditions the DMH treatment promotes the gel state of the membrane as more separations in the lipid phases of the membrane occurs in the bilayers of BBM vesicles, liposomes and plasma membranes of the colonic epithelial cells. Previous studies have demonstrated that the presence of phase separations in mixed phospholipid/cholesterol vesicles in which, a increase in initial fractional %Q was found to be associated with increasing cholesterol concentration. Since, in the present study also, cholesterol content in the colonic lipids and the BBM vesicles were found to be decreased more in the DMH group while it was increased in NSAIDs group, it can be speculated that the NSAIDs may induce the intrinsic lipid phase separation by perhaps regulating the cholesterol concentration in the membrane systems.

To study the polarity of the lipid interfaces, Laurdan (6-dodecanoyl-2-dimethylammonionaphthalene) was used, which is an amphiphilic fluorescent membrane probe synthesized by Prof G. Weber to study the dipolar relaxation processes. This probe has the advantage of displaying spectral sensitivity to the phospholipid phase state. Its steady state excitation and emission spectra can be resolved into two spectral components...
that correspond to the two phases. The time evolution of the Laurdan emission spectrum has been measured to detect the coexistence of different phospholipid phases in mixed vesicles and to determine the interconversion rate between the phases. Also, Laurdan excitation spectrum is different in the two phospholipid phases. Quantization of the relative amount of gel and liquid crystalline phases has been described by steady-state generalized polarization (GP) which is defined as:

$$GP = \frac{I_- - I_+}{I_+ + I_-}$$

Where $I_+$ and $I_-$ are the fluorescence intensities at the maximum emission in phospholipids in the gel and in the liquid crystalline phase, respectively (excitation GP or exGP). In addition, the GP value can be obtained using the fluorescence intensities at the maximum excitation in phospholipids in the gel and in the liquid crystalline phase (emission GP or emGP). The particular wavelength behavior of the GP value gives information on the coexistence of different phases and on their interconversion. The GP values are limited between +1 and - 1. The characteristic GP values in the pure gel (ordered) and in the pure fluid liquid-crystalline (disordered) membranes have previously been determined.11 Our result showed lower values of exGP and emGP for DMH treated group and increasing values with coxib treatments. The observed increase in exGP values in membranes of coxib groups indicated the presence of more lipids in gel phase as in the gel phase dipolar relaxation of the phospholipid is strongly reduced either because of the absence of water or because the phospholipid tight packing prevents a rapid relaxation of water molecules.12 Further, Paratassi et al.13 has shown that the Laurdan in different phospholipid vesicles has a well defined value for the gel phase (GP = 0.6) and for the liquid crystalline phase (GP = -0.2), respectively. In the present study taking the exGP values (fig. 3a) and emGP values (fig. 3b) into consideration it can be clearly seen that the control values are closer to the gel phase whereas for the DMH group values are closer to the liquid crystalline phase. In the coxib groups (Celecoxib and Etoricoxib) there is a shift in GP values towards the gel phase. These trends are better followed in case of the liposomal vesicles than in the BBM or colonicocyte vesicles probably because of more complexities involved in the nature and composition of the later membranes.14

Further, in the present studies, steady-state fluorescence polarization of lipid soluble probe was applied to assess membrane state of fluidity in terms of rotational diffusion as assessed by order parameter (using DPH). The particular usefulness of these methods stems from the fact that the polarization of the fluorescence of a molecule depends upon its rate of rotation.15 Hence, changes in rotation rate due to interactions of the probe with other species in the environment are readily observed and quantified. Since the rotation rate depends on the resistance offered by the microenvironment to the motion of the probe, fluorescence polarization provides an estimate of the environmental resistance which is interpretable as an apparent "microviscosity". From the DPH fluorescence measurements of order parameter, it can be seen that DMH group has lower order parameter indicating lesser phospholipid packing in the membrane bilayer. NSAIDs on the other hand have resulted in increasing the membrane lipid order and therefore, are responsible for creating more of the membrane into gel phase as also found with the Laurdan studies.

In the biological membranes phospholipids have a complex composition, including differences in length and unsaturation of their acyl chain residues and in the type of polar heads. Gel phase is assumed to be "less polar" with respect to the liquid crystalline phase and therefore, provides a good basis for the observed induction in lipid phase separations (as evident from the NBD-PE studies) in the NSAID groups. The converging effects of these changes in the membrane physical state was observed more evidently in terms of fluidity (fig. 4b), which was found to be more in DMH group (also indicated by higher phase separations or low polarity, and liquid crystalline phase state) and lesser for NSAID groups (as also indicated by higher phase separations or low polarity, and more of the gel phase). In addition, cholesterol and cholestrol: PL ratio is an important chemical index of membrane fluidity. The decrease in the chot: PL ratio as observed in the DMH group correlates with an increase in local polarity, fluidity and molecular disordering (as seen in terms of lesser phase separation and lower order parameter). From these results, it seems that DMH induces significant alterations at the membrane structural level by modulating its composition and alters its physical dynamics so as to make the conditions more favorable for the process of carcinogenesis. whereas, NSAIDs may cause their membrane modulating effects by affecting the lipid phase separations and thereby causing the decrease in membrane polarity.16 The concomitant increase in the number of apoptotic cells in NSAID treated groups in comparison to DMH group substantiates the anti-proliferative effects of NSAIDs. Indeed, in our previous study, within the colonic cell membranes NSAIDs have been found to affect many membrane associated processes (such as sodium proton exchange activity, intracellular pH, calcium homeostasis, mitochondrial dysfunction) and also responsible for the inhibition of membrane associated enzymes like cyclooxygenase, thereby, creating the microenvironment favorable for the inhibition of cellular progression of the colon cancer. The inhibition of COX was believed to be the sole explanation for the chemopreventive actions of NSAIDs. However, it is now believed that there should be additional mechanisms to explain the overall chemopreventive pharmacological behavior of NSAIDs. Since membrane dynamics are responsible for the functioning of membrane bound enzymes and permeability of the ions, the present study provides a possible explanation for the
ability of NSAIDs to influence these biophysical parameters contributing towards the induction of apoptosis during colon carcinogenesis. Results presented in this study can be highly relevant in providing some insight into the usefulness of NSAIDs as a potential chemopreventive agent.

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