Failure of Indomethacin To Inhibit Growth of the R3230AC Mammary Tumor in Rats

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ABSTRACT—The relationship between the dietary lipid-induced growth of the R3230AC mammary tumor and prostaglandin E2 (PGE2) levels as well as the effect of the prostaglandin synthetase inhibitor indomethacin (Ind) on these parameters was examined. F344 rats fed a high-fat (HF) diet containing 20% corn oil demonstrated more rapid tumor growth and higher tumor and plasma PGE2 levels than rats fed a 20% hydrogenated cottonseed oil (HCTO) diet. Addition of 0.004% Ind to the HF diet markedly reduced tumor and plasma PGE2 levels. However, Ind had no effect on tumor growth. Neither the fatty acid composition nor the insulin-binding capacity of the tumor plasma membranes was affected by Ind. Membranes from animals fed HF diets with or without Ind bound more 125I-labeled insulin than membranes from HCTO-fed rats. The results suggest that, for the R3230AC mammary tumor, reduction in both tumor and plasma PGE2 levels by Ind did not result in reduced tumor growth in animals fed diets high in polyunsaturated fatty acids.—JNCI 1985; 75:751-756.

The role that PG play in the increased rate of tumor growth seen in animals fed diets high in PUFA has received considerable attention in recent years. Since such diets contain a high percentage of linoleic acid, a precursor for PG biosynthesis, it has been suggested that ingestion of such diets would lead to increased synthesis of PG. One proposed mechanism for PG to increase tumor growth is by suppression of the immune system. PG have been shown to suppress cell-mediated immunity (1-3), and treatment with PG synthetase inhibitors overcome this suppression (4, 5). Diets high in PUFA were reported to inhibit lymphocyte function (6) and to stimulate PGE2 production (7) while enhancing tumor growth. In addition, inhibition of PG synthesis by Ind was found to reduce tumor growth in animals fed high PUFA-containing diets (7-9). A direct relationship between growth of the R3230AC mammary tumor and tumor PGE2 content was observed (10, 11). In contrast to these studies, other studies have found that Ind did not inhibit tumor growth (12-14) while significantly reducing PGE levels (12, 13) and that administration of anti-PGE antibodies led to the inhibition of cell-mediated immunity (15).

In the studies presented here, we sought to relate the effects of Ind treatment on growth of the R3230AC mammary tumor with plasma and tumor PGE2 content. It was reported previously that this transplantable tumor grew more rapidly in rats fed a high PUFA diet relative to its growth in rats fed either a high saturated fat or an FF diet (8, 16). In addition, the insulin-binding capacity of the tumor plasma membranes was examined to determine whether the previously observed relationship between tumor growth and insulin binding was maintained. The data obtained suggest that no simple relationship existed between R3230AC tumor growth and PGE2 levels.

MATERIALS AND METHODS

Animals, tumors, and diets.—Female F344 rats (75-90 g) were obtained from the Charles River Breeding Laboratories, Wilmington, MA. The R3230AC mammary tumor was implanted sc in the axillary region on both sides by a sterile trocar technique as described by Hilf et al. (17). Three days prior to tumor implantation, animals were placed on an HF diet, an HF diet plus 0.004% Ind (HF + Ind diet), or an HCTO diet (final concentration, <0.1% linoleic acid) (table 1). We added Ind to the diet by dissolving the Ind in a small volume of 99% ethanol (~8 mg Ind/ml ethanol) and then dis-
solving this mixture in the corn oil. The concentration of Ind selected was based on the report of Hillyard and Abraham (8). All diets, which were offered ad libitum, had an equivalent ratio of protein calories to total calories.

Animals were decapitated at 19 days after tumor transplantation. Animals from the different diet groups were pair sacrificed; i.e., 5 animals per week per diet group were sacrificed at the same time. The design of this experiment equalized variations due to transplantation of tumors from different weekly donors and provided more representative results. This protocol allowed us to prepare membranes from fresh, rather than frozen, tumors. At the time of sacrifice, blood was collected in 15×100-mm plastic tubes, which contained 100 μl Ind/BHT ethanol solution (0.1% Ind and 0.02% BHT in 99% ethanol) and 300 μl 4.5 mM EDTA in saline. This procedure prevents changes in PGE2 levels (18).

Tumors were dissected from animals at room temperature, and 1-g portions of tumor tissue were snap-frozen in liquid nitrogen and stored at −76°C until analysis was performed. This procedure was accomplished in less than 1 minute. The collected blood samples were centrifuged for 10 minutes at 1,000×g; plasma was removed and stored at −76°C.

**PG extractions and PGE2 determinations.**—PG were extracted from tumor tissue and plasma by the method of Powell (19). An Ind-BHT ethanol solution was used instead of ethanol in the extractions from tumor homogenates. Sep Pak columns (10×10 mm) were purchased from Waters Associates, Inc., Milford, MA. 125I-labeled PGE2 was added to selected portions of tumor homogenates and plasma samples, and the percent recovery was determined to be 71.0±1.2 and 73.1±7.8 from tissue and plasma, respectively. PGE2 levels were measured by radioimmunoassay, with the use of the PGE2 125I-labeling radioimmunoassay kit purchased from New England Nuclear Corporation, Boston, MA. The sensitivity of the assay was 0.25 pg PGE2, and the cross-reactivity for PGE1 was 3.7% and less than 0.4% for 12 other similar prostanoid species. The values of PGE2 presented were not corrected for recovery.

**Tumor membrane preparation and analysis.**—Plasma membranes were prepared essentially by the method of Shin et al. (20). Freshly excised tumors were homogenized in 4 volumes of ice-cold 0.9% NaCl, an equal volume of ice-cold 0.9% NaCl was added, and the homogenate was centrifuged at 4°C at 1,000×g for 10 minutes. The layer of fat at the top was removed, and the supernatant was collected. The pellet was resuspended in an equal volume of 0.9% NaCl, homogenized again with 15 strokes in a loose-fitting Dounce homogenizer, and centrifuged; the resulting supernatant was combined with the previously collected supernatant. The pooled supernatants were centrifuged at 4°C at 33,000×g for 7.5 minutes, and the resulting supernatant was collected and centrifuged at 4°C at 96,000×g for 75 minutes to yield a microsomal pellet. This pellet was resuspended in HEPES buffer (pH 7.4), containing 25 mM HEPES, 10 mM NaHCO3, 125 mM NaCl, 3 mM K2HPO4, 1 mM MgSO4, 1 mM CaCl2, and 11 mM glucose by passage through a 26-gauge needle attached to a syringe; the resuspended material was then mixed with an equal volume of 66% (wt/wt) sucrose. The final sucrose concentration was adjusted to 40% (wt/wt) by appropriate addition of either 66% sucrose or HEPES buffer as determined by a refractometer. The sample was fractionated by flotation through a step gradient containing 25, 33, and 37% sucrose (wt/wt) at 96,000×g at 4°C for 7 hours with the use of an SW27 rotor. The material at the 25-33% sucrose interface was collected, diluted with 3 volumes of 10 mM Tris-HCl (pH 7.4), and centrifuged for 1 hour at 4°C at 96,000×g. Membranes were then suspended in a small volume (≈1 ml) of HEPES buffer by being mixed in a syringe to which a 26-gauge needle was attached. The membrane suspensions were kept at 0°C until use.

Protein was determined by the method of Lowry et al. (21). 5′-Nucleotidase activity was assayed as described by Morre (22). This assay was done to assess the similarity of the membrane preparations. The 5′-nucleotidase specific activity per milligram protein was the same for plasma membranes prepared from tumors of animals fed the HF, HF + Ind, and HCTO diets (9.40±0.35, 10.1± 0.20, and 9.66±0.14 μmol Pi/mg protein/hr, respectively). The membranes typically exhibited a twelvefold enrichment of 5′-nucleotidase activity relative to the 5′-nucleotidase activity in the homogenate, compared to a fourteenfold enrichment reported by Shin et al. (20) for the same tumor. Plasma membranes were stored at −76°C until analyzed for fatty acid composition. Lipids were extracted by the method of Folch et al. (23). Extracted lipids were methylated and analyzed by GLC according to the modification detailed by Cave and Erickson-Lucas (24) with the use a 6-foot glass column packed with 10% SP-2330. Samples were also submitted to Dr. Charles Sweeley, Michigan State University, for analysis (GLC analysis on a 50-m DB-1 bonded phase capillary column). Identities of the fatty acids were presented were not corrected for recovery.

**Table 1.**—Diet composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Diet and composition, g/kg</th>
<th>HF</th>
<th>HF + Ind</th>
<th>HCTO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ind</td>
<td>—</td>
<td>40±4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Corn oil</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>—</td>
</tr>
<tr>
<td>Hydrogenated cottonseed oil</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>200</td>
</tr>
<tr>
<td>Casein (vitamin free)</td>
<td>230</td>
<td>230</td>
<td>230</td>
<td>—</td>
</tr>
<tr>
<td>Sucrose</td>
<td>145</td>
<td>145</td>
<td>145</td>
<td>—</td>
</tr>
<tr>
<td>Dextran</td>
<td>175</td>
<td>175</td>
<td>175</td>
<td>—</td>
</tr>
<tr>
<td>Rogers Harper vitamin mix</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>Rogers Harper salt mix</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin B12, 10 μg/ml</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>Choline chloride, 0.20 g/ml</td>
<td>15±4</td>
<td>15±4</td>
<td>15±4</td>
<td>—</td>
</tr>
</tbody>
</table>

*mg/kg.

**mg/ml/kg.
determined by comparison to known standards. Due to the lack of an available standard for C21:1 (No. of C-atoms: No. of double bonds), we tentatively assigned its identity on the basis of the linear relationship obtained by plotting the log of retention time versus carbon chain length of standard monounsaturated fatty acids for both chromatography systems, as well as the appearance of a peak comigrating with a C21:0 standard subsequent to reduction by H2 of the sample. Unequivocal identification C21:1 by mass spectrometry was not possible due to impurities in the sample.

Insulin binding to plasma membranes.—Crystalline porcine insulin was labeled stoichiometrically with Na125I with the use of chloramine-T according to the method of Freychet et al. (25). The labeled insulin had a specific activity of 60–260 μCi/μg and was greater than 98% trichloroacetic acid precipitable.

The specific binding of 125I-labeled insulin was measured as follows: We incubated 90–150 μg membrane proteins with 125I-labeled insulin in 250 μl HEPES buffer containing 5 mg bovine serum albumin/ml and 1 mg bacitracin/ml to minimize insulin degradation. Binding of 125I-labeled insulin was performed in duplicate in 1.5-ml conical microfuge tubes at final insulin concentrations of 10−8–10−14 M. After each assay, duplicate samples were also incubated in the presence of 10−6 M unlabeled insulin to obtain and then correct for nonspecific binding. The contents of the tubes were mixed; after a 1-hour incubation at room temperature (21°C), 1 ml ice-cold 0.9% NaCl was added, and the tubes were centrifuged for 4 minutes in a Beckman 3200 Microfuge. Membranes were washed once again with 1 ml ice-cold 0.9% NaCl, and the tubes were drained, blotted, and placed in a Beckman 8000 gamma counter (Beckman Instruments, Inc., Fullerton, CA) for measurement of radioactivity.

Measurement of serum insulin.—Insulin was measured by radioimmunoassay with an Insulin-MAIA Kit purchased from Serono Laboratories, Randolph, MA.

Data analysis.—The data were evaluated statistically by Student’s t-test; one-tailed and two-tailed analyses were used as appropriate, and P < .05 was considered to be significant.

RESULTS

Effects of Ind and Diet on Tumor Growth

The effects of various diets and Ind on tumor weights, body weights, and tumor burdens are summarized in table 2. Rats fed the HCTO diet had significantly lower body weights and tumor weights than rats fed the HF diets with or without Ind. Comparison of tumor burden, which accounts for differences in body weight, was considered to be a more meaningful indication of dietary-induced effects on tumor growth. Tumor burden in HCTO-fed rats was lower than that in HF-fed rats (P = .068) as well as significantly less than tumor burden in HF + Ind-fed rats (P < .025). Ind treatment had no effect on tumor burden or on tumor or body weight.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Ind, % in diet</th>
<th>Tumor wt, g</th>
<th>Body wt, g</th>
<th>Tumor burden, tumor wt/100 g body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF</td>
<td>—</td>
<td>4.32±0.59</td>
<td>144.5±2.8</td>
<td>3.04±0.41</td>
</tr>
<tr>
<td>HF</td>
<td>0.004</td>
<td>4.50±0.47</td>
<td>142.1±0.5</td>
<td>3.12±0.32</td>
</tr>
<tr>
<td>HCTO</td>
<td>—</td>
<td>2.86±0.38</td>
<td>124.3±2.6</td>
<td>2.27±0.27</td>
</tr>
</tbody>
</table>

aThere were 15 rats in each diet group.
bSignificantly less than HF diet (P < .025) and significantly different from HF + Ind diet (P < .02).
cSignificantly less than HF diet and significantly different from HF + Ind diet (P < .01).
dSignificantly different from HF + Ind diet (P < .05).

PGE2 Levels

The effects that diet and Ind treatment had on tumor and plasma PGE2 levels were assessed (table 3). The results indicate that Ind was very effective in reducing both tumor and plasma PGE2 levels. PGE2 content of the tumor was reduced by 89% and by 84% in plasma in animals fed the HF + Ind diets versus those fed the HF diet. Animals fed the HCTO diet had lower tumor and plasma PGE2 levels than HF-fed animals, although the reduction in tissue PGE2 content was not statistically significant (P = .107). However, the HCTO-fed animals had higher PGE2 levels than animals fed the HF + Ind diet. This result suggests that although the HCTO diet contains little or no PG precursors, body stores of these compounds were probably not completely depleted, leading to synthesis of PG.

Insulin Binding to Tumor Plasma Membranes

Specific 125I-labeled insulin binding to tumor plasma membranes was determined to explore the earlier proposal of a relationship between insulin binding and tumor growth (16). This was performed on membranes prepared from rats fed the various diets with the use of a range of 4 × 10−11 to 10−8 M insulin (table 4). The data indicate that membranes prepared from rats fed either the HF or the HF + Ind diet bound equal amounts of insulin; i.e., Ind treatment had no effect on insulin-binding capacity. However, insulin binding to mem-

<p>| Table 2.—Effects of Ind and diet on growth of the R3230A C tumor |
|---------------------------|-------------|--------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Diet</th>
<th>Ind, % in diet</th>
<th>Means ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>HF</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>HCTO</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

aValues are means ± SE, and numbers in parentheses represent the No. ofsamples assayed.
bSignificantly less than HF diet (P < .025) and HCTO diet (P < .05).
cSignificantly less than HF diet (P < .01) and HCTO diet (P < .025).
dSignificantly less than HF diet (P < .05).
bran samples from both groups of HF-fed rats was significantly higher than that to membrane samples from HCTO-fed rats (P < 0.01). We attribute these differences in binding to alterations in the number of membrane-binding sites, as determined by Scatchard analysis of the curvilinear plots (not shown). The estimated numbers of binding sites were 5.36, 5.90, and 3.85 X 10^9/100 /lg protein, and the dissociation constant values were 1.64, 1.70, and 1.48 nM for membranes from the HF-, HF + Ind-, and HCTO-fed rats, respectively. These results are consistent with our previous results suggesting a relationship between growth of the R3230AC tumor and insulin-binding capacity of the plasma membranes (16).

Plasma insulin levels were measured to assess their possible influence on the insulin-binding capacity of the membranes, i.e., down regulation of the insulin receptor. The plasma insulin levels were found to be comparable in animals fed the three dietary regimens: 38.5±4.3, 41.0±4.7, and 39.4±3.1 μU (± SE)/ml for 6 random samples for rats fed the HF, HF + Ind, and HCTO diets, respectively.

### Fatty Acid Composition of Mammary Tumor Plasma Membranes

The effects of Ind treatment and diet on the plasma membrane fatty acid composition of the R3230AC tumor are shown in table 5; data are presented as percent of total lipid. Comparisons of membrane samples from the HF-fed animals indicate that there were no differences. Ind treatment, which inhibits the conversion of arachidonic acid (C20:4) to PG (27, 28), could be expected to cause an accumulation in PG precursors in the membrane. This, however, was not the case. Differences in the fatty acid composition between membranes from the HCTO- and HF-fed animals were similar to those reported earlier (16). Membranes from rats fed the HF diets had significantly higher proportions of the polyunsaturated fatty acids (C18:2 and C20:4) and significantly lower percentages of the monounsaturated fatty acids (C18:1 and C21:1) than membranes from animals fed the HCTO diet. A lower percentage of C16:1 appeared to occur in membranes from the HF-fed animals, although this difference was not statistically significant.

### DISCUSSION

Growth of the R3230AC transplantable mammary tumor was reported to be more rapid in rats fed diets high in PUFA than in rats fed diets devoid of these fats (8, 16). In these studies we examined whether this faster growth rate could be inhibited by the PG synthetase inhibitor Ind and whether a relationship was demonstrable between tumor growth and PGE2 levels. The results presented here indicate that Ind treatment had no effect on tumor growth in animals fed an HF diet, although PGE2 tumor and plasma levels were greatly reduced in the Ind-treated animals. However, a comparison between the HCTO- and the HF-fed rats suggested the existence of a relationship between PGE2 levels and tumor growth; animals fed the HCTO diet had lower plasma and tumor PGE2 levels along with slower tumor growth. On the basis of these observations, we conclude that no simple relationship exists between R3230AC tumor growth and PGE2 levels.

Our failure to observe an effect of Ind on tumor growth is similar to what others have seen for B16 melanomas in mice (12, 13) and BP 8/P, tumors in mice (14). Ind failed to inhibit growth of both of these tumors; for the B16 melanoma the rate of development of palpable subcutaneous tumors was hastened (13), and for the BP 8/P tumor Ind treatment yielded tumors that contained more viable tumor cells, as assessed by trypan blue exclusion methods (12). In contrast to those studies,
other studies have found that Ind inhibited the dietary lipid-induced growth of 7,12-dimethylbenz[a]anthracene-induced tumors (9) as well as the 3910-50 adenocarcinoma (8) and DMBA-4 transplantable tumors (7). Using the R3230AC tumor, Karmali (10) observed (unpublished observations) that tumor growth was inhibited in animals treated with Ind.

This apparent discrepancy of Ind action on tumor growth may be explained by the suggestion made by Mertin and Stackpole (15) that the effect of PG on the immune response follows a bell-shaped dose-response relationship. These authors observed that the administration in vivo of anti-PGE antisera inhibited cell-mediated immunity. According to their hypothesis, low PG concentrations would be required to initiate or enhance the induction of cell-mediated immune responses, whereas high concentrations would inhibit the process. Studies examining PG levels in relation to tumor growth would lend support to their postulation. Inhibition of R3230AC tumor growth by glutathione (10) and omega-3 fatty acids (11) was accompanied by a 44% and an average of 41% reduction in PGE2 tumor content, respectively. Studies that reported no effect of Ind on tumor growth reported decreases in tumor PGE2 content of 66% (14), greater than 80% (13), and 89% in the data presented here. Animals in this study fed the HCTO diet had 38% less tumor PGE2 content and slower tumor growth as compared to the HF-fed animals. It, therefore, appears that reductions of tumor PGE2 content by approximately half were correlated with inhibition of tumor growth, whereas greater reductions in PGE2 content were not accompanied by reduced tumor growth. However, the values reported for PGE2 levels in R3230AC tumors differed. Karmali and co-workers (10, 11) reported control values (laboratory chow-fed rats) of 88 and 16 ng PGE2/g tumor and 20 ng PGE2/g tumor in animals fed 5% coconut oil diets (29).

Our results for PGE2 levels in R3230AC tumors are in general agreement with the results from two of the aforementioned reports. Since no data on PGE2 levels in R3230AC tumors from Ind-treated rats were presented (10), it is not possible to ascertain whether reductions in PGE2 levels, similar to those reported here, were observed in those experiments. The question remains whether it is the absolute level of tumor PGE2 or the proportional decrease that may relate to tumor growth behavior. Whether a similar relationship exists for plasma PGE2 levels and/or other PG is unknown, although Karmali (10) observed that inhibition of R3230AC tumor growth by glutathione resulted in an increase in PGE1, PGE2α, 6-keto-PGF1α, and thromboxane B2.

We have previously demonstrated that growth of the R3230AC tumor in intact rats may be related to the insulin-binding capacity of the tumor plasma membranes, on the basis of the faster growth and higher insulin-binding capacity of membranes from HF- versus FF-fed rats (16). One limitation of that study was that the animals fed the FF diet had significantly higher serum insulin levels than the HF-fed animals, and although the differences in serum insulin levels were modest, it was not possible to preclude that they contributed to the observed differences in insulin binding, i.e., down regulation of the insulin receptor. In this report all animals were fed diets containing equal amounts of fat and carbohydrate, and animals from the 3 diet groups presented with the same serum insulin levels. Under these circumstances we also observed a relationship, similar to our previous findings, between insulin-binding capacity and tumor growth; membranes from animals fed either the HF or the HF + Ind diet bound more insulin than membranes from the HCTO-fed animals.

Since the precursors for PG biosynthesis arise from the fatty acids of phospholipids in the cell membrane (30), we analyzed the relative proportions of these precursors in the tumor plasma membranes. Analysis of fatty acids indicated that, although there were differences between membranes from HCTO- and HF-fed rats (16), Ind treatment had no effect on the fatty acids in the plasma membrane. This finding is similar to the results obtained by Rao and Abraham (31), who observed only a minimal effect of aspirin on the fatty acid composition of a transplanted mouse mammary tumor. Since Ind has been shown to inhibit the conversion of arachidonic acid to PG (27, 28), one might have expected an accumulation of arachidonic acid in the plasma membranes. However, increased synthesis of other arachidonic acid metabolites, e.g., leukotrienes, hydroperoxy fatty acids, and hydroxy fatty acids, by the lipoxygenase pathway may explain why inhibition of the above metabolic pathway did not result in increased precursor concentrations (30).

The results presented here suggest that if a relationship exists between PG and R3230AC tumor growth, it is a complex one, in which both high and low levels of PGE2 result in similar rates of tumor growth.

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