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Influence of Esculetin on Incidence, Proliferation, and Cell Kinetics of Mammary Carcinomas Induced by 7,12-Dimethylbenz[α]anthracene in Rats on High- and Low-fat Diets

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The effects of a high-fat diet and esculetin were investigated on 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis in female Sprague-Dawley rats. Rats were given a 5-mg dose of DMBA. Seven days later, they were fed either a high-fat (20% soybean oil) or low-fat (0.5% soybean oil) diet. A half of the rats received diets containing 0.03% esculetin. Esculetin significantly inhibited tumor incidence, growth and cell kinetics of the tumor in the rats fed the high-fat and the low-fat diets. Our findings indicate that DMBA-induced mammary tumorigenesis is affected by lipoxygenase products.

Key words: Esculetin — High dietary fat — Mammary carcinoma — 7,12-Dimethylbenz[*a*]-anthracene

Eicosanoids are metabolites of arachidonic acid, a fatty acid that is synthesized from dietary linoleic acid. An association between fat intake and breast cancer in humans has been suggested,[1–3] while total calories have also been proposed as a factor which potentially influences breast cancer development.[4,5] A diet high in linoleic acid was associated with an increase in mammary carcinogenesis in rodents.[6] Although the exact mechanism of the effect of dietary fat has not been identified,[6] arachidonic acid-derived eicosanoids[7] are believed to play an important role. The role of arachidonic acid metabolites in mammary carcinogenesis has been examined by the use of various inhibitors of arachidonic acid metabolism.[7–18] Most experiments have focused on inhibitors of the cyclooxygenase pathway, but some of these studies have produced inconsistent results.[11,16–18] Herein, we report the effects of esculetin, a lipoxygenase inhibitor,[19] on 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis in Sprague-Dawley (SD) rats fed high- or low-fat diet.

Fifty-day-old inbred virgin female SD rats received a single 5-mg dose of DMBA (Sigma Chemical Co., St. Louis, MO) via an intragastric tube. Seven days later, the rats were switched from laboratory chow to either a high-fat (20% soybean oil) or low-fat (0.5% soybean oil) diet (Oriental Yeast Co., Tokyo). A half of the rats in both the low-fat and high-fat groups were given a diet supplemented with 0.03% (w/w) esculetin (Sigma) (Table I). The constituents of high-fat and low-fat diets were as reported elsewhere,[17] except for the use of soybean oil instead of corn-oil in this study. The diets were formulated on the assumption that the rats would consume an equal number of calories, and equal amounts of vitamins and minerals. However, the high-fat diet also had a high-fiber content. Esculetin was premixed with the vitamins and minerals; the mixture was then added to the other diet ingredients. The feed was stored in sealed plastic containers at 4°C in the dark. Food and water were available *ad libitum* until 20 weeks after DMBA administration.

Body weight and tumor incidence and size were recorded weekly throughout the experimental period. Tumor location was determined by palpation and the size was measured with a vernier caliper in two perpendicular dimensions. Estimated tumor weight (ETW) was calculated by means of the following equation[20]: ETW = largest diameter × shortest diameter^2/2 (mg). Initial tumor weight was defined as the average ETW of the first palpable tumors in the tumor-bearing rats in each group. Average tumor weight was defined as the average ETWs of all tumors in tumor-bearing rats in each group. The cumulative tumor weights were calculated by summing the average ETWs of all tumors in tumor-bearing rats in each group. Tumor doubling time (*Td*) was calculated by use of the following equation[21]: $Td = t\ln 1/2(\ln Vt - \ln VO)$, where t is the time period between tumor appearance and termination of the experiment; Vt is ETW at the termination of the experiment, and VO is the ETW at tumor appearance. Tumor incidence was assessed in terms of the average percent tumor incidence, and the average number of tumors in a rat for all rats in each group and for tumor-bearing rats in each group. In

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Table I. Effects of High Dietary Fat and Esculetin on Tumorigenesis of DMBA-induced Mammary Carcinoma in Rats

Group	No. of rats	No. of rats with tumors	Tumor incidence (%)	No. of tumors	No. of tumors in a rat[a]	No. of tumors/tumor-bearing rat[b]	Letency period (wk)
HF[c]	15	13	87[d,e]	39	2.6±2.2[f,g]	3.0±2.1[d]	8.6±5.5[d]
HF+Esculetin	15	8	53	19	1.2±1.5	2.4±1.1	11.7±4.7
LF	15	8	53	11	0.7±0.8	1.4±0.5	13.8±5.7
LF+Esculetin	15	3	20	4	0.3±0.6	1.3±0.6	11.7±1.2

a) The average number of tumors per rat for all rats in each group.
b) The average number of tumors per rat for tumor-bearing rats in each group.
c) HF, high-fat group; HF+Esculetin, esculetin-treated high-fat group; LF, low-fat group; LF+Esculetin, esculetin-treated low-fat group.
d) $P<0.05$ versus LF.
e) $P<0.05$ versus HF+Esculetin.
f) $P<0.01$ versus LF.
g) Mean±SD.

addition, the average latency period to tumor appearance (the interval between DMBA administration and the appearance of the first palpable tumor) was calculated for tumor-bearing rats in each group. Twenty weeks after DMBA administration, all rats in each group were injected intraperitoneally with 20 mg/kg of bromodeoxyuridine (BrdUrd) (Sigma). The animals were killed 1 h later, and all palpable tumors were removed. Each tumor was fixed immediately in 10% formalin. Two 5-μm thick sections of each tumor were obtained from paraffin tumor blocks. One section was stained with hematoxylin-eosin for histological examination. The second section was deparaffinized in xylene and then rehydrated in a graded series of ethanol. Endogenous peroxidase activity was blocked by immersing the sections in methanol containing 0.3% hydrogen peroxide for 15 min. After being washed in distilled water for 5 min and in phosphate-buffered saline (PBS, pH 7.4) for 20 min, the sections were incubated in 2 *N* HCl for 30 min, neutralized with 0.1 *M* $Na_2B_4O_7$ for 10 min, and rinsed three times in PBS for 5 min. The sections were then incubated with 10% normal goat serum (DAKO, Copenhagen, Denmark) in PBS with 0.1% bovine serum albumin (BSA) for 15 min at room temperature and then incubated overnight at 4°C with an antibody against BrdUrd (Becton Dickinson Immunocytometry Systems, San Jose, CA) at a 1:50 dilution in PBS-BSA. After being rinsed in PBS, sections were incubated with biotinylated antimouse immunoglobulin (DAKO) for 60 min at room temperature and then rinsed with PBS. For visualizing antibody binding, sections were incubated with a streptavidin-biotin-peroxidase complex (DAKO) for 60 min at room temperature, and then immersed in Tris-buffered saline (pH 7.4) containing 3,3′-diaminobenzidine and 0.1% hydrogen peroxide for 3 min and counterstained with 0.1% hematoxylin. The sections were then rinsed in tap water for 5 min, dehydrated in ethanol, rinsed in xylene, and mounted in malinol medium (Muto Pure Chemicals, Tokyo). Negative controls were prepared by omitting the primary antibody. For each specimen, the number of nuclear-stained tumor cells in 500 cells was counted under a microscope at a magnification of ×400. The BrdUrd lebeling index was expressed as the average number of all tumors for tumor-bearing rats in each group.

Total body weight did not differ significantly among the groups throughout the experimental period. Mammary tumors were histologically identified as adenocarcinomas. The high-fat diet significantly increased the incidence of mammary tumors compared with the low-fat diet ($P<0.05$). Esculetin significantly inhibited tumor incidence in the high-fat diet groups ($P<0.05$) (Table I, Fig. 1). Latency period was significantly shorter in the high-fat diet group than in the low-fat diet group, but it was not significantly different among the high-fat diet groups or among the low-fat diet groups (Table I). Twenty weeks after DMBA administration, initial tumors were significantly larger in the high-fat diet group than in the low-fat diet group (Table II). Esculetin inhibited tumor proliferation in the high-fat diet group: the initial tumors in the esculetin-treated high-fat diet group were significantly smaller than those in the high-fat diet group ($P<0.05$). There was no significant difference in ETW among the two low-fat diet groups. Initial tumors in the esculetin-treated low-fat group tended to be larger than those in the low-fat diet group, but the number of tumors wes too small to permit analysis. Tumor doubling time was not significantly different among the groups, but the BrdUrd labeling indices were significantly higher in the high-fat group than in the

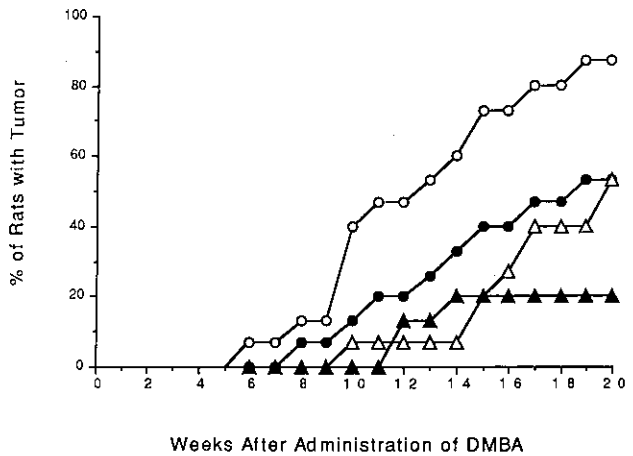

Fig. 1. Effects of esculetin on the cumulative incidence of palpable mammary tumors in rats fed high- or low-fat diet. ○, high-fat; △, high-fat plus esculetin; ●, low-fat; ▲, low-fat plus esculetin.

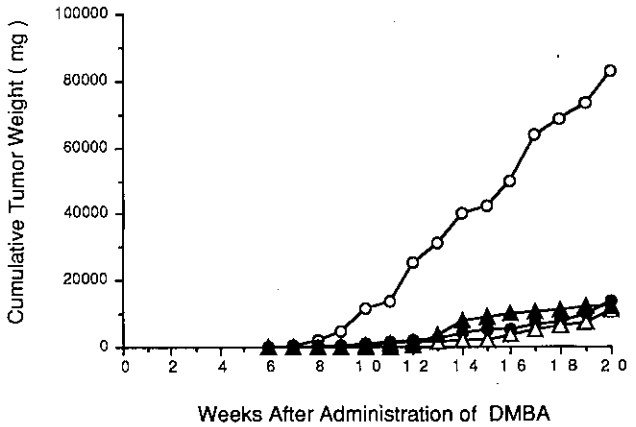

Fig. 2. Effects of esculetin on the cumulative ETW of palpable mammary tumors in rats fed high- or low-fat diet. ○, high-fat; △, high-fat plus esculetin; ●, low-fat; ▲, low-fat plus esculetin.

Table II. Effects of High Dietary Fat and Esculetin on Tumor Growth, and Cell Kinetics of DMBA-induced Mammary Carcinoma in Rats

Group	Initial tumor weight[b] (g)	Avg. tumor weight[c] (all tumors) (g)	Tumor doubling time (days)	BrdUrd labeling index (%)
HF[a]	2.89±2.00[d, e, g]	6.37±5.14[d, e]	30.2±18.6	10.8±4.3[e, f]
HF+Esculetin	1.08±1.11	1.73±1.41	39.1±15.2	7.4±4.0
LF	1.17±0.94	1.46±1.26	36.4±22.0	3.7±1.8
LF+Esculetin	2.66±1.20	4.00±1.69	46.2±8.4	2.6±2.0

a) HF, high-fat group; HF+Esculetin, esculetin-treated high-fat group; LF, low-fat group; LF+Esculetin, esculetin-treated low-fat group.
b) Average estimated tumor weight of the first tumor of each rat at 20 weeks after DMBA administration.
c) Average estimated tumor weight of all tumors in tumor-bearing rats in each group.
d) $P<0.05$ versus LF.
e) $P<0.05$ versus HF+Esculetin.
f) $P<0.01$ versus LF.
g) Mean±SD.

low-fat diet group ($P<0.01$). Esculetin significantly reduced the BrdUrd lebeling indices in the high-fat diet group ($P<0.05$) (Table II). Esculetin was associated with a reduction in cumulative ETW in both high-fat and low-fat diet groups (Fig. 2).

Arachidonic acid may be metabolized through the cyclooxygenase pathway, resulting in prostaglandin production, or through the 5-lipoxygenase pathway, resulting in production of leukotrienes and 5-hydroxyeicosatetraenoic acid. Multiple interactions among products of the arachidonic acid cascade have been reported.[22] Although most studies that have examined the role of arachidonic acid metabolites in mammary tumorigenesis and their effects on the immune response have focused on cyclooxygenase-derived eicosanoids,[7-18] the results of some of these studies are inconsistent.[11, 16-18] In an *in vivo* study, Carter *et al.*[16] reported that although the cyclooxygenase inhibitors carprofen and indomethacin similarly reduced prostaglandin E_2 levels in the serum and the mammary epithelium, only indomethacin inhibited DMBA-induced mammary carcinogenesis. Previously, we found that indomethacin significantly reduced tumorigenesis in rats fed a high-fat diet, but significantly promoted tumor proliferation in rats fed either a high-

or a low-fat diet.[17, 18] Indomethacin is primarily an inhibitor of the cyclooxygenase involved in prostaglandin synthesis, but at higher concentrations, it also inhibits phospholipase A_2[23] and 5-lipoxygenase, which are required for leukotriene synthesis.[24] Therefore, it would be of interest to determine whether esculetin, a lipoxygenase inhibitor, would affect mammary carcinogenesis.

Esculetin is an inhibitor of lipoxygenase enzymes but may actually stimulate prostaglandin production.[19] Lipoxygenase inhibitors are generally considered to have more potent effects than cyclooxygenase inhibitors on tumor proliferation,[25] but information is limited concerning the effects of esculetin on mammary tumorigenesis. Lee and Ip[26] recently reported that esculetin inhibited cell proliferation of the TMT-081 rat mammary tumor cell line in a concentration-dependent manner in association with an increase in cyclooxygenase products *in vitro*. Rose and Connoly[27] found that esculetin suppressed cell proliferation of MDA-MB-231 breast cancer cells *in vitro*. Evidence suggests that leukotrienes B_4, C_4 and D_4, which are products of the 5-lipoxygenase pathway, may stimulate growth of malignant cells,[28, 29] while Snyder *et al.*[30] found that leukotrienes B_4 and C_4 stimulated DNA synthesis but failed to reverse the inhibition of growth induced by piriprost. In the present study, esculetin markedly inhibited the proliferation of mammary tumors in rats fed a high-fat diet, although the result was inconclusive in rats fed a low-fat diet. Since esculetin inhibits 5- and 12-lipoxygenase, our findings suggest that lipoxygenase products of arachidonic acid may affect mammary tumorigenesis. Although indomethacin stimulated tumor proliferation in DMBA-induced mammary carcinoma in previous studies,[17, 18] this effect may have been related to increased lipoxygenase activity.[22, 31–33]

In conclusion, our findings indicated that DMBA-induced mammary tumorigenesis was affected by lipoxygenase products. However, additional studies will be required to clarify the changes in the eicosanoid profile that occur in response to cyclooxygenase and/or lipoxygenase inhibitors and diets that alter mammary tumorigenesis.

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