Potent preventive action of curcumin on radiation-induced initiation of mammary tumorigenesis in rats

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Introduction

Tumor initiation by radiation in mammary glands is dependent upon cell stage, because estrogen is a direct or indirect sensitizer for tumor initiation by radiation (1,2). Previous studies in our laboratory have demonstrated that administration of aminothiols, such as S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721) and cysteamine, prior to irradiation has a potent preventive effect at the initiation stage of mammary tumorigenesis (3). The protection against radiation offered by WR-2721 (4) and cysteamine (5) is considered to be due to the scavenging of free radicals produced by the interaction of biological molecules and radiation. WR-2721 and cysteamine are toxic at effective doses (6,7), therefore, we have undertaken an evaluation of less toxic phytochemicals whose anti-oxidant properties may make the potential chemopreventive agents for radiation-induced mammary tumorigenesis. A recent study has indicated that phytochemicals with anti-oxidant and anti-inflammatory properties can inhibit tumor initiation and promotion in mouse skin (8). 1,7-Bis(4'-hydroxy-3'-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin), a major pigment in turmeric obtained from the powdered rhizomes of Curcuma longa L., possesses both anti-inflammatory and anti-oxidant properties (9) and has no toxicity (10). In the 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor model, when rats were fed a diet containing 1% curcumin prior to dosing with the chemical carcinogen, the incidence of animals with tumors was not significantly altered (11). Because radiation is the only proven relevant human breast carcinogen, we have attempted to evaluate the preventive effects of curcumin on radiation-induced initiation of mammary tumorigenesis and on estrogen-induced tumor promotion in rat mammary glands initiated with radiation in our research series. Our previous study suggested that when administered orally for a long period, curcumin has potent preventive activity during tumor promotion in radiation-initiated mammary tumorigenesis (12). In the present study we have carried out further investigations of the chemopreventive effects of dietary curcumin on radiation-induced initiation.

Materials and methods

Materials

Diethylstilbestrol (DES), cholesterol, and sulfatase were purchased from Sigma (St Louis, MO). β-Glucuronidase was purchased from Wako Pure Chemical Industries (Osaka, Japan). Pellets were prepared in a medical Silastic tube (Dow Corning, Midland, MI) and were filled with 3 mg DES mixed with...
H. Inano et al.

Fig. 1. Experimental schedule in this study. Open bar, control diet (MB-1); closed bar, diet containing 1% curcumin; closed arrowhead, whole body irradiation with 1.5 Gy γ-rays at day 20 of pregnancy for tumor initiation; open arrowhead, implantation with DES pellet for tumor promotion; M, months old.

27 mg cholesterol. Curcumin, commonly used in food as a coloring agent, was obtained from Aldrich Chemical Co. (Milwaukee, WI). Diet containing 1% (w/w) curcumin was prepared in biscuit form by Funabashi Farm (Chiba, Japan). A basal diet (MB-1) of the same form was used for the control experiments. The major components of MB-1 are as follows: total carbohydrate, 54.1%; protein, 24.6%; fat, 4%; fiber, 3.8%; moisture, 7.9%; ash, 5.8% [2,4,6,7-H]Estradiol-17β (sp. act. 4 TBq/mmol) was purchased from Du Pont/NEN Research Products (Boston, MA).

Animals and treatment

The rats used in the present study were treated and handled according to the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on the Safety and Handling Regulations for Laboratory Animal Experiments in our Institute. Wistar-MS rats from a stock colony of Nippon SLC Co. (Hamamatsu, Japan) were kept at 23 ± 1 °C in a controlled environment (14 h light/10 h dark). They received water and food ad libitum. For experiments on the prevention of mammary tumors, 54 female rats, 2.5 months old, were mated and then randomized into two groups of 27 rats each at day 11 of pregnancy (the presence of a vaginal plug denoting day 1). The control rats were fed a basal diet (MB-1) throughout the experimental period, received whole body irradiation with 1.5 Gy γ-rays (0.15 Gy/min) from a 60Co source at day 20 of pregnancy and were implanted with a DES pellet at 1 month after weaning (Figure 1). The experimental group rats were fed the diet containing 1% curcumin between day 11 of pregnancy and parturition (day 23 of pregnancy) and were implanted with a DES pellet at 1 month after termination of nursing. The pellets were replaced every 8 weeks. The rate of release of DES from the pellet was 0.38 ± 0.01 μg/day (13). The rats were examined for palpable mammary tumors for 1 year starting from the date of pellet implantation. When mammary tumors >2 cm in diameter were detected, the rats were killed by CO2 asphyxiation and the tumors were removed for further observation. Each mammary tumor was fixed in 10% neutral buffered formalin for histopathological examination. The remaining rats were killed 1 year after administration of the DES pellet and were autopsied to ascertain whether they had any non-palpable mammary tumors and pituitary tumors. Tumor incidence was calculated from the number of rats that developed tumors within 1 year. Iball’s index of mammary tumors was calculated as follows: the ratio of incidence (%) to the average latency period in days×100 (14). For studies on the morphological and biochemical effects of the treatment with curcumin, separate experiments were carried out for which 20 pregnant rats were divided into two groups of 10 rats each at day 11 of pregnancy. Control rats were fed the basal diet and the curcumin group the diet containing 1% curcumin. At day 20 of pregnancy, corresponding to the time of irradiation for tumor initiation described above, six rats in each group were killed for biochemical and morphological studies. The remaining four dams in each group bore pups at full-term gestation. Body weights of dams and newborn pups were measured after parturition.

Assays

A blood sample, collected from each rat by cardiocentesis under anesthesia, was allowed to clot and was centrifuged to obtain serum. The sera were immediately frozen and stored at −80°C until the assay was started. Concentrations of prolactin, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were determined with NIDDK radioimmunoassay kits (the National Hormone and Pituitary Program, Rockville, MD). The serum concentrations of estradiol-17β and progesterone were assayed with commercially available radioimmunoassay kits. For assays of total curcuminoids (free form plus conjugates), serum was incubated with 10 nM McIlvaine buffer (pH 5.0) containing 20% ascorbic acid, 0.17% EDTA, 500 U β-glucuronidase and 40 U sulfatase at 37°C for 60 min (15). Curcumin and its metabolites were extracted with ethylacetate and then analyzed by HPLC with a multilength detector on a Develosil ODS-HG-5 column (4.6×250 mm; Nomura Chemical Co., Seto, Japan) eluted with a mixture of acetonitrile/water (1:1 v/v) containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The chromatogram was monitored at a wavelength of 430 nm for detection of curcumin and at 280 nm for tetrahydrocurcumin (16). Fatty acids were extracted from serum with hexane and then were treated with 14% trifluorobenzene dissolved in methanol: methanol:benzene (35:30:5 v/v/v) for 10 min in boiling water for esterification. The methyl esters of fatty acids were analyzed by gas chromatography with a hydrogen flame ionization detector (17). For assay of lipid peroxidation products, the serum was mixed with 20% trichloroacetic acid and 0.67% thiobarbituric acid and heated for 15 min in boiling water. The concentration of thiobarbituric acid-reactive substances (TBARS) was calculated from the number of rats that developed tumors within 1 year. Iball

References

Statistical analysis

Statistical analyses were conducted using the χ2 test for incidence of mammary tumors and for the proportion of adenocarcinoma and fibroadenoma and Student’s t-test for the level of significance of the difference between two mean values of body weight, organ weight, and fatty acid concentrations, latent period and multiplicity. The cumulative proportions of rats with tumors (incidence curves) were calculated by the product-limit method where rats which died or were killed without mammary tumors were included and the difference between groups was tested for statistical significance by the Mantel–Cox test. The analyses were performed using StatView-J4.5 software (Abacus Concepts, Berkeley, CA). P values <0.05 were considered significant.
The proportion of adenocarcinoma and fibroadenoma in the control group was 50% of that in the curcumin-fed group (Table I). In the curcumin-fed group, the proportion (16.7%) of adenocarcinoma was decreased to 50% of that in the control group. Conversely, the proportion (83.3%) of fibroadenoma was 2-fold higher than that in the control group. However, no significant difference ($P = 0.450$) in the proportion of adenocarcinoma and fibroadenoma was observed between the two groups with the $\chi^2$ test.

**Biological effects at the time of initiation of curcumin administration during pregnancy**

Body weight of dams was decreased to 91% of that observed in rats fed the control diet by administration of the curcumin diet from day 11 of pregnancy, in spite of a similar intake of diet throughout the experiment. No change in weight of liver ($P = 0.262$), adrenal gland ($P = 0.378$) or pituitary gland ($P = 0.079$) of dams at day 20 of pregnancy was observed between the control and curcumin-fed groups (Table II). The litter size of curcumin-fed rats was comparable with that of the rats fed the control diet. In addition, the body weight of fetuses at day 20 of pregnancy was increased slightly by the administration of curcumin, but no significant difference was observed ($P = 0.109$). Also, the body weight (5.6 ± 0.1 g) of pups (1 day-old) born to curcumin-fed dams was the same as that of pups of the dams fed the control diet (5.6 ± 0.1 g).

**Biological effects at the time of initiation of curcumin administration during pregnancy**

Serum concentrations of ovarian and pituitary hormones were measured 10 days after the start of the administration of curcumin, the time corresponding to initiation with radiation. No significant differences in estradiol-17β ($P = 0.677$) and progesterone ($P = 0.223$) concentrations were observed between the two groups (Table III). In addition, curcumin did not have any effect on the concentrations of prolactin ($P = 0.502$) and FSH ($P = 0.883$). However, the concentration of LH in the rats fed the curcumin diet was increased to 1.8-fold of that observed in rats fed the control diet ($P < 0.05$). No significant difference ($P = 0.592$) in TBARS was observed on administration of curcumin for 10 days. The serum concentration of curcumin in rats fed the curcumin diet was below the level detectable by HPLC (4 ng/ml). The tetrahydrocurcumin concentration was 39 ± 10 ng/ml in the curcumin-fed rats.
Histological observations of and number of ER in mammary glands at the time of initiation

Whole mounts of inguinal mammary glands corresponding to the time of irradiation were prepared to examine the effects of curcumin on development and differentiation of the glands in pregnant rats. On day 20 of pregnancy, mammary glands of rats fed the control diet showed many alveolar buds with branched lactiferous ducts (Figure 3a). The whole mounts showed that the mammary glands in pregnant rats fed the curcumin diet exhibited the same development as the glands of control rats (Figure 3b). On histological examination, no significant differences were observed in the population of parenchymal cells (the glandular epithelium) in the glands of pregnant rats fed the curcumin diet compared with those in control rats (Figure 3c and d). These observations at the time of irradiation were consistent with the finding of no significant differences in the number of ER in the mammary glands of control (10.9 ± 1.0 fmol/mg protein) and curcumin-fed rats (10.0 ± 0.5 fmol/mg protein) (P = 0.466).

Effect of curcumin on the fatty acid profile in serum at the time of initiation

The serum concentrations of fatty acids were measured 10 days after the start of administration of dietary curcumin, corresponding to the day of irradiation. No significant change was observed in the concentrations of the fatty acids assayed (Table IV). With regard to unsaturated fatty acids, the serum concentrations of linoleic acid (P = 0.487), linolenic acid (P = 0.628) and arachidonic acid (P = 0.093) were slightly decreased by the administration of dietary curcumin. However, the concentrations of eicosapentaenoic acid (P = 0.912) and docosahexanoic acid (P = 0.505) were weakly increased in rats fed the curcumin diet. Ratios of total saturated fatty acids to total unsaturated fatty acids (S/U) and of total saturated fatty acids to total polyunsaturated fatty acids (S/P) were not significantly altered by 10 days administration of curcumin.

Biological effects at the end of the curcumin treatment experiment

The final body weights and organ weights are summarized in Table V. No significant changes in body weight were observed between the control and curcumin-fed groups (P = 0.253). Treatment with curcumin during initiation by radiation decreased the liver weight (P < 0.01) and increased the uterus weight (P < 0.05) significantly. When the 27 control rats were autopsied, eight (29.6%) were found to have developed pituitary tumors; the pituitary tumor incidence (18.5%) in the curcumin-fed rats was two-thirds of that in the control rats (P = 0.340) (Table I). No change in weight of normal pituitary...
glands \((P=0.389)\) and pituitary glands with tumors \((P = 0.493)\) was observed between the control and curcumin-fed groups.

**Discussion**

Explanations of the cytotoxic effects of radiation have previously emphasized the involvement of reactive oxygen species such as the superoxide anion \((\text{O}_2^\cdot)\) and the hydroxyl radical \((\text{OH})\) \((22,23)\). McLemman et al. have reported that \(\text{O}_2^\cdot\) may be non-toxic, but it is a precursor in the formation of \(\text{OH}\), which is the most toxic radical resulting from radiation \((24)\). The involvement of oxygen-derived free radicals in the carcinogenic process correlates well with the protective effects of free radical scavengers, as seen by inhibition of the development of radiation-induced mammary tumors by administration of WR-2721 prior to irradiation \((3,25)\). A recent study indicated that reactive oxygen radical species generated by radiation increased the frequency of a tandem \(\text{CC} \rightarrow \text{TT}\) double substitution in the DNA strand \((26)\).

Chemo prevention is a rapidly growing field in cancer research which focuses on inhibiting and delaying the onset of carcinogenesis. A large number of natural products have been evaluated as potential chemopreventive agents \((27,28)\). Recent studies on components of plants indicated that phenolic compounds with anti-oxidant and/or anti-inflammatory properties can inhibit tumor initiation and promotion in mouse skin \((8)\). Most of the natural anti-oxidants have either a phenolic group or a \(\beta\)-diketone group \((29,30)\). Curcumin is a unique compound, having both phenolic and \(\beta\)-diketonic functional groups, and would be expected to have remarkable anti-oxidant and free radical scavenging activities \((31,32)\). Curcumin not only exhibits the above properties, but also enhances the activities of anti-oxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase \((33)\). Furthermore, curcumin is a potent inhibitor of oxygen radical-generating enzymes such as cyclooxygenase-2 \((34,35)\).

The formation of chromosomal aberrations \((36)\) and micronucleated polychromatic erythrocytes \((37)\) caused by whole body exposure to \(\gamma\)-rays were significantly inhibited by oral administration of curcumin. Also, curcumin suppressed lipid peroxidation in rats irradiated with \(\gamma\)-rays \((38,39)\). Earlier studies from our laboratory demonstrated a marked preventive effect of curcumin on DES-dependent promotion in radiation-initiated mammary tumorigenesis \((12)\). The data presented herein indicate that administration of curcumin for 12 days, i.e. 9 days before and 3 days after irradiation, also markedly reduced radiation-induced initiation in mammary tumorigenesis in rats. Curcumin has been shown to display anti-initiation activities, as indicated by its ability to prevent tumorigenesis induced in the colon by azoxymethane \((40)\), mouth by 4-nitroquinoline-1-oxide \((41)\), skin by benzo[a]pyrene \((42)\) and duodenum by \(N\)-ethyl-\(N\’-nitro-\(N\)-nitrosoguanidine \((43)\). It was suggested that many chemical carcinogens act by forming free radicals \((44-46)\). We would suggest that one possible mechanism of the anti-initiation activity of curcumin is the scavenging of free radicals produced by a variety of chemical carcinogens or radiation as tumor initiator at target sites. However, dietary curcumin did not lower the cumulative incidence or affect tumor multiplicity in the initiation stage of DMBA-induced mammary tumorigenesis \((11,47)\). The reason why no protective effect of curcumin was observed in the chemical carcinogenesis of mammary glands is still not known.

Nitric oxide plays a key role in physiological as well as pathological processes, including inflammation and cancer. The enhancement of NO production by irradiation was attributed to high levels of expression of inducible nitric oxide synthase \((\text{iNOS})\) \((48)\). Excessive production of NO by activated iNOS may result in the formation of toxic intermediates, such as peroxynitrite \((\text{ONOO}^-)\) and \(\text{N}_2\text{O}_5\), causing tissue damage and genotoxicity \((49,50)\), and thus has potential carcinogenic effects \((51)\). In immunohistochemical experiments, iNOS expression was apparently increased in the basal layers of alveoli and lactiferous ducts of the mammary glands treated with lipopolysaccharide \((\text{LPS})\) as an inflammatory agent and this increase was reflected in an enhancement of NO production \((52)\). Furthermore, NO production by LPS-stimulated mammary glands was significantly decreased in the presence of curcumin, as was the amount of a 122 kDa iNOS \((53)\). On the other hand, 3,5,4\’-trihydroxy-trans-stilbene \((\text{resveratrol})\), a phyto-phenol isolated from the seeds and skins of grapes, inhibited the expression of LPS-induced iNOS \((54)\) and decreased LPS-stimulated NO production \((55)\). Mbongoye et al. \((56)\) have reported that resveratrol is a potential chemopreventive agent for both ER-positive and ER-negative breast cancers. Also, formation of azoxymethane-induced colonic aberrant crypt foci was significantly suppressed in the presence of an iNOS-specific inhibitor, \(S,S’-1,4\)-phenylene-\(bis\-(1,2-\text{ethanediyl)bis-isothiourea})\) \((57)\). These findings suggest that suppression of iNOS activity by curcumin in the mammary gland of irradiated rats helps to prevent radiation-induced tumor initiation.

The transcription factor nuclear factor \(\kappa B\) \((\text{NF-}\kappa B)\) has been implicated in the inducible expression of a variety of genes involved in inflammatory and immune responses \((58)\). Singh and Aggarwal have reported that curcumin inhibits the NF-\(\kappa B\) activation pathway at a step before inhibitory protein \(\kappa B\) \((\text{I}kB)\) \(\alpha\) phosphorylation \((59)\). Recently, Jobin et al. \((60)\) have reported that interleukin \((\text{IL})-\beta\)-mediated expression of the adhesion molecule, intercellular adhesion molecule-1, and the chemokine IL-8 were reduced by blockade of transcriptional activation cascades, such as cytokine-induced NF-\(\kappa B\) DNA binding activity, RelA nuclear translocation, IkB\(\alpha\) degradation, IkB\(\alpha\) Ser32 phosphorylation and IkB kinase activity, by curcumin. Their results suggest that curcumin blocks a signal upstream of the NF-\(\kappa B\)-inducing kinase, but below the junction of the IL-1\(\beta\) signal pathways. NF-\(\kappa B\) is activated by radiation \((61,62)\). Activation of NF-\(\kappa B\) may be particularly important for cell survival in response to oxidative stress induced by radiation, but it has been shown recently that inhibition of NF-\(\kappa B\)-activation enhances radiation-induced apoptosis \((63,64)\). We would suggest that another possible mechanism of the chemopreventive activity of curcumin for mammary tumorigenesis is elimination of radiation-initiated tumor origin cells from the mammary gland by apoptosis.

At the time corresponding to initiation with radiation, no detectable serum curcumin was observed in rats fed the curcumin diet. It was shown that curcumin administered orally was metabolized to tetrahydrocurcumin during absorption through the intestine \((65,66)\). In fact, tetrahydrocurcumin was detected in serum of rats fed the diet containing curcumin for 9 days in the present study. Tetrahydrocurcumin exhibited a significant inhibitory effect on \(\text{O}_2^\cdot\) generation induced by 12-O-tetradecanoylphorbol-13-acetate \((67)\) and on lipid peroxidation of erythrocyte membrane ghosts induced by \(\text{t}-\text{butylhydroperoxide}\) compared with curcumin \((16,68)\). Also,
feeding of a diet containing tetrahydrocurcumin resulted in a significant repression of 1,2-dimethylhydrazine-induced formation of aberrant crypt foci, which are regarded as a precursor lesion for colon cancer (69). The results obtained from the present study thus suggest that tetrahydrocurcumin has potential as a chemopreventive agent for radiation-initiated mammary tumorigenesis.

Finally, Wahlström and Blennow (70) found no apparent toxic effects of curcumin at doses of up to 5 g/kg body wt in rats when given orally. In the present study, pregnant rats consumed 18.2 ± 0.3 g of diet containing 1% curcumin/day, which corresponds to 0.67 g curcumin/kg/day, for 12 days. Body weight in pregnant rats fed the curcumin diet was reduced to 91% of that observed in rats fed the control diet. The reduction was significant, but would be too low to indicate a toxic action of curcumin. Therefore, it is likely that a reduction in body weight occurs in curcumin-fed rats having a decreased concentration of serum triglycerides (12). Curcumin did not have any adverse effects on growth or teratogenesis of fetuses nor on organ weight or serum concentrations of hormones of dams, suggesting no toxic effect when administered orally. Lack of a mutagenic effect of curcumin was also reported in the presence or absence of a rat hepatic microsomal activation system in the Ames test with Salmonella typhimurium (71).

In conclusion, radiation-induced initiation of mammary tumorigenesis was markedly inhibited by administration of dietary curcumin. Oral administration of curcumin did not produce any side-effects on endocrinological and physiological status. These results raise the possibility of clinical application of curcumin in the management of radio-diagnosis to diminish tissue damage by radiation.

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