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Author's Profile

Dr. TAKUJI TANAKA, M.D., Ph.D., FIAC
1978 - 1983: Assistant Professor, Department of Pathology, Gifu University School of Medicine, Japan
1983 - 1985: Visiting Scientist, Division of Experimental Pathology and Toxicology, Naylor Dana Institute, American Health Foundation, New York, USA
1985 - 1997: Associate Professor, Department of Pathology, Gifu University School of Medicine, Japan
1997 - : Professor and Chairman, Department of Oncologic Pathology, Kanazawa Medical University, Japan

Specialty and Present Interest:
Inflammation and Cancer; Cancer chemoprevention by natural products; Biomarkers of carcinogenesis and cancer chemoprevention; Chemical carcinogenesis

Memberships for Academic Societies:
Japanese Cancer Association (1976-; Councilor, 2000-)
Japanese Society of Pathology (1976-; Councilor, 1986-)
Japanese Society of Toxicologic Pathology (1986-)
International Academy of Pathology (1987-)
International Academy of Cytology (MIAC, 1987-; FIAC, 1989-)
American Association for Cancer Research (1989-)
European Association for Cancer Research (1991-)

Editorial Board:
Journal of Carcinogenesis (Sr. Editor)
Molecular Carcinogenesis
Chemico-Biological Interactions
Asian Pacific Journal of Cancer Prevention
The Open Toxicology Journal
Journal of Toxicologic Pathology
Japanese Journal of Food Chemistry
Journal of Toxicological Sciences (Associate Editor)

Breast Cancer Chemoprevention: Current Perspectives

Takeru Oyama[1], Yumiko Yasui[1] and Takuji Tanaka*[,1,2]

[1]Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293 and [2]Tohkai Cytopathology Institute: Cancer Research and Prevention (TCI-CaRP), 4-33 Minami-Uzura, Gifu City, Gifu 500-8285, Japan

Abstract: The increasing knowledge regarding breast cancer carcinogenesis has provided possible opportunities to prevent breast cancer. This paper reviews the issues related to breast cancer chemoprevention including the identification of high-risk groups, biomarkers and potential chemopreventive agents. Trials with tamoxifen have clearly shown that the risk of developing estrogen receptor (ER)-positive breast cancer can be reduced at the later stage of this malignancy. However, there was no beneficial effect on ER-negative cancers. The challenge is to find new agents, which achieve same or better efficacy but with fewer side effects. Raloxifene has similar efficacy to tamoxifen, but leads to more favorable histologic profiles of endometrial tumors and fewer gynecologic symptoms and thrombo-embolic events. Adjuvant trials for contralateral tumors suggest that aromatase inhibitors may be able to prevent up to 70-80% of ER-positive breast cancers. This is currently being investigated in two large prevention trials, the IBIS-II trial using anastrozole and the MAP.3 trial using exemestane. We therefore need to discover novel agents for ER-negative breast cancer.

Keywords: Breast cancer, chemoprevention, prevention trials, tamoxifen, aromatase inhibitors, SERMs.

INTRODUCTION

There are 1.2 million new cases of breast cancer worldwide every year, which far exceeds the number of any other cancers, with the cervix now being a distant second at about 400,000 [1]. Not only is breast cancer the most common cancer in women, but it is also rapidly increasing, especially in the developing world. Breast cancer is thus the most common cancer in women world-wide and continues to be a major health problem [2]. In the USA, it was estimated that in the year 2001 192,000 women would be diagnosed with breast cancer and 40,200 will die of this disease [3]. In the UK, more than 44,000 women were diagnosed in 2004. Although the mortality is declining, the incidence continues to increase and therefore, prevention is an important aim. In Japan, breast cancer ranks the first cause of death due to malignant neoplasms for women and the incidence is increasing.

Several breast tumor markers were considered (Table 1). Some categories showed evidence of clinical utility and were recommended for use in practice: CA 15-3, CA 27.29, carcinoembryonic antigen, estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2, urokinase plasminogen activator/plasminogen activator inhibitor 1, and certain multiparameter gene expression assays. Not all applications for these markers were supported, however. The other categories demonstrated insufficient evidence to support routine use in clinical practice: DNA/ploidy by flow cytometry, p53, cathepsin D, cyclin E, proteomics, certain multiparameter assays, detection of bone marrow micrometastases, and circulating tumor cells.

Population-based programs, based on reducing obesity [4] and increasing exercise [5], are likely to be effective for breast cancer [2, 3] just as they have been for heart disease. Four prevention trials that used tamoxifen *vs.* a placebo in high-risk women have suggested an overall decrease of breast cancer of 38% [6, 7] and a considerable beneficial carry-over effect of tamoxifen has been reported [8]. Tamoxifen has a good therapeutic ratio in premenopausal women, but this is less true in postmenopausal women [9]. In many adjuvant trials, aromatase inhibitors (AIs) have been shown to be better than tamoxifen in preventing relapse, but also decrease the incidence of contra-lateral breast cancer by about 50% in comparison to tamoxifen [10]. These data provided the rationale for prevention trials that used AIs, such as the International Breast Intervention (prevention) Study (IBIS II), which compares 5 years of anastrozole treatment (1 mg/day) with a placebo in postmenopausal women at increased risk of breast cancer. Although the prevention of breast cancer is a worthy goal, this needs to be achieved with as little iatrogenic harm as possible in order for such treatment programs to be acceptable.

Table 1. Specific Tumor Markers of Breast Cancer

CA 15-3 and CA 27.29
Carcinoembryonic antigen
estrogen receptor and progesterone receptor
DNA flow cytometry-bases parameters (DNA content, S phase, or others)
Immunohistochemically based markers (HER-2)
p53
Urokinase plasminogen activator/plasminogen activator inhibitor 1
Catepsin D
Cyclin E
Proteomic analysis (proteomic pattern)
Multiparameter gene expression analysis (Oncotype DX assay)
Bone marrow micrometastases
Circulating tumor cell assays

Research in breast cancer now extends in many directions, from the identification of genes that predispose women to breast cancer, to cellular models for preneoplastic disease,

*Address correspondence to this author at the Tohkai Cytopathology Institute: Cancer Research and Prevention (TCI-CaRP), 4- 33 Minami-Uzura, Gifu City, Gifu 500-8285, Japan; Tel: +81-76-218-8116; Fax: +81-76-286-6926; E-mail: takutt@kanazawa-med.ac.jp

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the investigation of the tumor and its local environment and finally to the identification of risk factors for the development of breast cancer and possible models of breast cancer prevention, including the use of chemopreventive agents.

While risk factors only identify individuals most likely to develop a disease, a key requirement for a biomarker is that it responds to treatment in a way that quantitatively predicts the extent of risk reduction for an individual. At present, there are only candidate biomarkers for a few cancers, notably breast cancer and prostate cancer. Mammographic density is the most promising biomarker for breast cancer and more than 40 studies that date back to the original work by Wolfe [11] have shown an increased risk for women with radiographically dense breasts [12]. Since then other researchers [13] have shown that quantification of the proportional area of the breast that is covered by mammographic dense tissue is the best measure available. Further improvements can be expected in the measurement of density through the use of computerized assessments, volume measurement and identification of other radiologic features, such as diffuse disease *vs.* a nodular pattern, or structured densities. However, even using current techniques breast density is a common, readily measurable factor that indicates an appreciable increase in risk in both pre-menopausal and post-menopausal women [14, 15]. Although much remains to be learned about how changes in density affect risk, the fact that breast density is reduced by tamoxifen [16] and increased by hormone-replacement therapy [17] suggests that we predict the effect on risk from breast density.

This short review article summarizes recent advances of breast cancer prevention studies that include preclinical and clinical trials.

CARCINOGENESIS AND CHEMOPREVENTION OF BREAST CANCER

Carcinogenesis is characterized by a long delay between the first exposure to known or suspected carcinogen(s) and the eventual occurrence of cancer and by a steep rise in tumor incidence after this latency period. During this period, multi-step processes occur including tumor initiation, tumor promotion and tumor progression [18]. This multi-step nature of neoplastic development has been derived from several experiments on skin carcinogenesis [19] and from the proposed concept of tumor progression in mouse mammary carcinogenesis [20]. In general, newly acquired genetic alterations and molecular damage accompany the progression phase. The accumulation of these genetic alterations then provides the basis for the transition from a pre-malignant to a malignant state [18, 21].

Sporn and Newton [22] in 1979 defined chemoprevention as the prevention of cancer by the use of pharmacological agents that inhibit or reverse the process of carcinogenesis. In the process of carcinogenesis, altered states of cell/tissue differentiation are characteristic of premalignant lesions long before they become invasive, which therefore offers a window of time and a target for chemical intervention. It is possible to reverse the abnormal differentiation with an agent that is essentially non-cytotoxic [23]. Even though the precise mechanism of breast cancer carcinogenesis is not known, the terminal duct lobular units (TDLUs) might be the major stem cell component giving rise to premalignant breast lesions; ductal hyperplasia (DH), atypical ductal hyperplasia (ADD), ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) are thought to be histological manifestations of a continuum process leading from the premalignant to the malignant state [24]. Genetic alterations and molecular changes occur during this process and for effective breast cancer chemoprevention development, it is extremely important to identify specific molecular abnormalities that can be monitored as biological endpoint biomarkers during specific types of intervention.

RISK FACTORS OF BREAST CANCER

Epidemiological studies have identified several factors that are associated with an increased risk of developing breast cancer. One of the best known risk factors for breast cancer is family history. It is known that 5-10% of breast cancers are due to inherited genetic mutations [25]. Two of these genes, BRCA-1 and BRCA-2 have been cloned and, together, are estimated to be involved in 52% and 32% families of hereditary breast cancer, respectively [26]. Women with mutations of these genes have an approximate 50%-80% life time risk of developing breast cancer [27]. Germline mutations in the tumor suppressor gene p53 are also associated with increased breast cancer risk and account for 1% of breast cancers in young women [28]. However, it is important to note that most women with a family history do not have genetically inherited disease. Also it is important to distinguish this group from the genetically inherited group, since the former carries a lower life time risk of developing breast cancer. Epidemiological data also suggest an association between ovarian hormones and the risk of breast cancer. Indeed, prolonged estrogen exposure, such as early menarche [29], late menopause [30], nulliparity and late age at first pregnancy [29] were found to be associated with an increased risk of breast cancer. Recent data suggest that prolonged lactation can actually reduce breast cancer risk [31].

Multiple studies have described the relationship between the exogenous use of hormones and breast cancer risk. One meta-analysis revealed that the use of hormonal replacement therapy was associated with a small increased risk [32]. Another meta-analysis revealed essentially the same results [33]. Studies investigating the relationship between the use of oral contraceptives and breast cancer revealed that oral contraceptives slightly increased the risk of breast cancer, especially if they were used before the first birth [34].

Individuals with a histopathological diagnosis of proliferative breast disease have up to twice the normal risk of increased risk of breast cancer; the relative risk increases to 4.5-5.0, if atypia is present [35]. LCIS is also known to be a risk factor; specifically, the relative risk of LCIS patients for developing breast cancer has been estimated to be 7-9 times that of the normal population, with an absolute life time risk of 20% [36].

The evaluation of risk factors for breast cancer, mainly by epidemiological studies, has helped to develop models to identify women at increased risk. The most commonly used model is the Gail model, which used data from 4496

matched pairs of cases in the Breast Cancer and Diagnosis and Demonstration Project [37]. The risk factors for this model included the age, age at menarche, age at first live birth, number of previous breast biopsies, presence of atypia, and number of first-degree relatives with breast cancer. This model was validated by subsequent studies [38, 39]. Unfortunately, the model does not take into account the risk in individuals who have second-degree relatives diagnosed with breast cancer. It also underestimates the risk in individuals with a history of previous LCIS or DCIS and may overestimate the risk in women with non-proliferative disease at biopsy.

Dunning *et al.* [40] elegantly reviewed the genetic polymorphisms and breast cancer risk, but they did not include Japanese women. Miyoshi *et al.* described that the CYP 17 [41], CYP 19 (aromatase) [42] and CYP1A1 [43] polymorphisms would be useful for predicting breast cancer risk as well as some tumor characteristics in Japanese women.

INTERMEDIATE BIOMARKERS FOR BREAST CARCINOGENESIS

Intermediate biomarkers of cancer are phenotypic, genotypic and molecular changes that alter during carcinogenesis. Currently, there are no validated surrogate endpoint biomarkers for breast cancer in the context of chemoprevention trials with invasive cancer as the definitive end points. Hence, the development of intermediate biomarkers as surrogate endpoints for clinical chemoprevention trials for breast cancer is quite important. Because of the shorter latency to intermediate biomarker end points and the smaller cohorts required for treatment, planning short-term prevention trials and evaluating potential biomarkers in this setting is therefore critical to making progress in the chemoprevention of cancer, including breast cancer. To be most useful, intermediate biomarkers should be on the causal pathway of breast cancer and reflect biological changes along with the carcinogenic process, so that they can be used to monitor the efficacy of any type of breast cancer preventive intervention [44]. Even though endogenous and exogenous estrogen promotes the development of breast cancer, early initiating events should initially take place to sensitize breast epithelial cells to growth factor regulated cancer promotion. Cells in the breast epithelium of high-risk patients may comprise a pool of genetically altered cells that are subject to growth factor-mediated cancer promotion.

Epigenetic alterations, including hypermethylation, could reflect the presence of DNA-damaged cells. Methylation is the main epigenetic modification in humans [45] and is one of the mechanisms for inactivating tumor suppressor genes [46]. In particular, the hypermethylation of normally unmethylated CpG islands in many tumor suppressor genes correlates with loss of expression [45]. Abnormal methylation in breast cancer has been observed in the tumor suppressor genes p16 [47], BRCA1 [48], estrogen receptor (ER) [49], progesterone receptor [50], retinoic acid receptor (RAR)-β2 [51] and E-cadherin [46]. Methylated markers could thus potentially serve as biomarkers to identify high-risk women, as well as intermediate endpoint markers in short-term chemoprevention trials [52]. The centrosomal serine/threonine kinase 15/breast tumor amplified kinase (STKISBTAK), which plays a role in chromosomal instability, could also serve as a marker for an altered genetic status [53]. Amplification of the STK15/BTAK gene and overexpression of STK15/BTAK mRNA has been indeed reported in human breast cancers [54], indicating that it might be involved in breast cancer carcinogenesis.

Imbalances in proliferation and apoptosis, by favoring the promotion of genetically altered cells in the breast tissue, most probably play a role in breast cancer promotion. In fact, apoptosis occurs regularly during normal growth and development of the mammary gland. One of the most dramatic examples of apoptosis is evident during the remodeling of the breast that accompanies post-lactational involution. In transgenic mouse models, the over-expression of some growth factors, such as epidermal growth factor (EGF), are able to block this remodeling and serve as survival factors for the mammary epithelium [55]. The over-expression of epidermal growth factor receptor (EGFR) is considered to be important autocrine stimulatory pathway for breast cancer cell growth and its expression is associated with an enhanced metastatic potential in model systems [56]. Interestingly, an abnormal ploidy and EGFR expression are observed in benign breast epithelium in women who later develop breast cancer [57].

p53, which serves a multi-functional role as a transcriptional regulator, mediates G1-S growth arrest and plays a critical role in maintaining DNA integrity by facilitating the apoptosis of DNA-damaged cells. Mutations of p53 are present in up to 50% of invasive breast cancers and its loss of function is associated with a high proliferation index and poor clinical outcome [58]. In most instances, p53 abnormalities become appreciable just before the transition from pre-invasive to invasive disease [59]. In a case control study with more than 4,800 women, the presence of p53 mutations in benign epithelium is found to be an early marker for later breast cancer development [60]. Another proliferative factor is Her-2/neu, which is over-expressed in about 30% of breast cancers [61]. The activation of the Her-2/neu oncogene triggers a cascade of growth signals that results in gene activation [62]. Its over-expression is associated with loss of ER, aggressive clinical behavior, increased metastatic potential and altered sensitivity to hormonal and chemotherapeutic agents [63]. Recently, Her-2 is reported to exert its proliferative effect also by increasing cyclooxygenase (COX)-2 expression [64]. In addition, COX-2 inhibition blocks the proliferative effect of Her-2 [64]. Another survival and proliferation factor is COX-2, which is believed to play an important role in breast carcinogenesis [65-68]. COX-2 expression is present in up to 88% of all invasive breast cancers [69]. COX-2 expression has also been reported to increase in DCIS [65]. COX-2 *via* increasing prostaglandin (PG) levels, is able to stimulate the transformation and growth of mammary epithelial cells [70]. Mammary gland involution is delayed in COX-2 over-expressing transgenic mice, with a decrease in the apoptotic index of mammary epithelial cells [71]. Furthermore, COX 2 inhibitors may be specific inhibitors of normal epithelial cell proliferation and growth of malignant cells [72]. Therefore, COX-2 could be a potential marker with which to follow different types of chemopreventive intervention and it might also serve as a target itself for selective COX-2 inhibitors [68]. There is data suggesting an

interaction between COX-2 and the aromatase pathway in the breast epithelium. The aromatase is activated by COX [73], thus leading to estrogen-induced proliferation. Aromatase expression is the highest in or near the breast tumor [74], thus suggesting that aromatase is involved in the autocrine and paracrine mechanisms of initiation and progression of breast cancer. Insulin-like growth factor (IGF)-I and IGF-II are potent epithelial mitogens that stimulate the growth of human breast cancer cells and prevent apoptosis [75, 76]. IGFs are also involved in the later stages of progression and invasion by enhancing cancer cell migration [77]. These effects are mediated through the IGF-I receptor (IGF-IR), which is over-expressed in most breast cancer cell lines and in malignant breast tissues [78]. In addition, the circulating IGF-I levels are elevated in patients with breast cancer in comparison to healthy women. More importantly, increased levels of IGF-I are associated with subsequent development of premenopausal breast cancer [79]. In addition, stamoxifen is reported to modulate serum IGF-I [80]. This suggests that it has a potential to act as an intermediate endpoint marker for chemoprevention trials. Another proliferation marker, Ki-67, has been reported to increase in breast tissue with ductal hyperplasia, which is adjacent to invasive breast cancer [81]. Ki-67 could thus serve as an early proliferation marker for monitoring chemopreventive drug effects. While the G1 to S transition is regulated by cyclin E in normal dividing cells [82], cyclin E expression is elevated and associated with death and/or relapse from breast cancer [83].

The published literature is awash with examples of new tissue biomarkers promising to predict responses to therapy in breast cancer patients. However, few, if any, of these progress from the laboratory to the clinic [84]. There is currently great interest in the detection and characterization of putative precursor breast cancer lesions because of the possibility of chemoprevention. Knowledge of the biologic features of premalignant lesions, although limited, is rapidly evolving. Premalignant breast lesions have been examined for the presence of genetic alterations and for the expression of biomarkers such as ER, Ki67, p53, and HER2/neu [85]. Candidate biomarkers for clinical application within the next few years are topoisomerase II alpha, epidermal growth factor receptor, AKT, phosphatase, tensin homologue, autoantibodies and abnormal tumor-specific DNA methylation found in cell-free plasma DNA [84, 86]. Breast density was increased among premenopausal women with high levels of IGF-I and low levels of IGFBP3 which is consistent with the observed effect of these molecules on breast cancer risk [87]. In the future, it is likely that a combination approach to simultaneously measure multiple markers would be most successful in detecting early breast cancer. Ideally, such a biomarker panel should be able to detect breast cancer in asymptomatic patients, even in the setting of normal mammogram and physical examination results.

CHEMOPREVENTIVE AGENTS AND CLINICAL TRIALS

Since the target population for primary breast cancer prevention is high-risk but otherwise healthy individuals, much emphasis needs to be placed on the development of active but non-toxic chemopreventive agents. The agents which are currently in use as well as potential agents are discussed below.

Selective Estrogen Receptor Modulators (SERMs)

The term selective ER modulator (SERM) is recently used to describe compounds that interact with the ER but have tissue-specific activities [88]. A variety of compounds classified as anti-estrogens are known to have both estrogen-agonistic and estrogen-antagonistic properties [89].

Tamoxifen, one of the SERMs, is a triphenylethylene derivative with both estrogenic and anti-estrogenic activities [90]. Tamoxifen was first shown to prevent new contralateral tumors in women with breast cancer in 1985 [91]. This and the findings of supporting animal studies [92, 93] led to the proposal to use this drug in primary prevention of high-risk women [94]. Four prevention trials have now been completed. The combined results of these trials [95] indicated that about half of ER-positive tumors can be prevented with 5 years of prophylactic tamoxifen, but this agent has no impact on ER-negative women. Overall this amounts to a 38% reduction in the risk of breast cancer.

On the other hand, there were two major side effects of tamoxifen - increases in endometrial cancer and venous thrombo-embolic events during the active treatment phase. The former is increased about 2.5-fold whereas the latter is approximately doubled. In simple terms giving 5 years of tamoxifen to 1,000 women aged 50 at double the population risk would lead to 11 fewer breast cancers, six additional deep vein thromboses and three extra endometrial cancers in the first five years of follow-up. Given that breast cancer is the most serious of these events, the balance appears to be reasonably favorable.

However, a key question will be the extent to which benefits and side effects extend beyond the 5 year treatment period. Recent studies [8, 96] show that the benefits extend well beyond the active treatment period, but the side effects largely do not. In particular in years 5-10, after 5 years if tamoxifen in the IBIS-I trial, the risk of new ER-positive breast cancer was reduced by 44%. In addition, endometrial cancer and thrombo-embolic events were not in excess after completion of treatment. Therefore, one can expect that another 11 cancers will be prevented in this period and there will be no additional major side effects, so that the 10 year risk-benefit ratio will be substantially improved over the 5 year estimate currently available. Furthermore, since there is no diminution of benefit even at year 10, the benefits could persist even longer, making tamoxifen chemoprevention even more attractive, especially for women in the late premenopausal years, where life-expectancy is long.

Raloxifene, another SERM, is a nonsteroidal benzothiophene compound, chemically distinct from tamoxifen and estradiol that binds to ER to competitively block estrogen-induced DNA transcription in the breast and endometrium [97]. Four trials have reported on the use of raloxifene for breast cancer prevention. Two independent parts of the MORE/CORE trial have reported on the reduction of breast cancer in osteoporotic women. The original intent of this trial was to reduce bone fracture rates [98]. After 4 years of treatment a 65% reduction in all breast cancer was found in

the MORE segment [99]. This led to another 4 years of blinded treatment in the CORE study, where breast cancer was the primary endpoint. The results in that study were also very favorable with a 50% reduction in breast cancer [100]. Raloxifene appears to be associated with some increase of thrombo-embolic complications, as with tamoxifen, but it does not stimulate the endometrium, and therefore no increase in the number of endometrial cancers or other gynecologic problems is observed.

The RUTH study, which evaluated the impact of raloxifene on cardiovascular endpoints in 10,101 women at increased risk of cardiovascular events [101] found reductions in breast cancer similar in size to that seen for tamoxifen in other studies. Also the STAR trial comparing raloxifene directly to tamoxifen in 19,747 women at high-risk for breast cancer recently found similar efficacy for the two drugs in preventing invasive breast cancer, but fewer gynecologic and thrombo-embolic side effects with raloxifene [102]. Based on these results, one can safely anticipate that raloxifene will become a useful part of the armitarium for preventing post-menopausal breast cancer.

Other newer generation SERMs, such as toremifene, droloxifene, idoxifene, LY 353,381.HCL, EM 652 and faslodex (ICI 182,780) either have been or are currently being developed for the treatment of breast cancer and are also potential agents for chemoprevention [103]. Other promising agents include selective ERα and ERβ modulators, which will have tissue-specific activity and a favorable toxicity profile [104].

Aromatase Inhibitors (AIs)

Most of what we know about the potential use of AIs in prevention derives from adjuvant studies in women with early breast cancer, where the development of isolated contra-lateral tumors as a first event is a good model for prevention of new tumors in healthy women. This has proved to be a reliable source for estimating the qualitative effects of tamoxifen in prevention, both in terms of the major side effects and the efficacy. This approach has generally been more reliable than animal models or observational epidemiologic studies; although randomized intervention studies in the prevention setting remain essential for directly quantifying effectiveness in this setting and balancing risks and benefits. To date, eight different adjuvant trials have reported on the use of three different AIs for postmenopausal women with breast cancer [22-29]. In these trials, adjuvant AIs have been found effective in three clinical settings, as initial treatment, after 2-3 years of tamoxifen, or as extended treatment after 5 years of tamoxifen. To date, 8 different adjuvant trials have been reported on the use of these different AIs for postmenopausal women with breast cancer [105-110]. In these trials, a consistent reduction in the rates of contra-lateral breast cancer has been observed in the group receiving the AI. For example, in the ATAC trial, the number of contra-lateral breast cancers was reduced from 59 in the tamoxifen arm to 35 on anastrozole, a 42% reduction (95% CI, 12-62%; P = 0.01). A larger reduction of 53% (95% CI, 27-71%; P = 0.001) was seen in the hormone receptor-positive patients [109]. Tamoxifen itself is known to reduce the incidence of contra-lateral tumors by 46% in women with mostly ER-positive primary tumors, suggesting that the overall reduction of receptor-positive breast cancer associated with anastrozole in comparison to no treatment may be around 70-80%. Information on the receptor status of the secondary cancers in this trial is not yet available, but one would expect the preventive effect to be restricted to ER-positive contra-lateral tumors and to be greater for this group than for new breast tumors overall. The profound estrogen depletion associated with AIs produces a novel physiological state and this is bound to have other effects beyond those related to breast carcinogenesis. These effects can most reliably be studied in prevention trials where a placebo is employed, allowing a direct determination of the effect of the AI. There have been suggestions from adjuvant trials comparing AIs to tamoxifen, that AIs may also reduce endometrial cancer to below baseline rates, but a thorough evaluation is difficult because there is no untreated comparison group. Bone loss leading to increased fracture rates appear to be the most serious side effect of AIs and methods for addressing will be essential if these drugs are to be used prophylactically [111]. Adherence problems with AIs are mainly due to arthralgias.

Retinoids

Retinoids represent a promising group of agents for the prevention of breast cancer. Fenretinide, a synthetic amide of retinoic acid, induces apoptosis through mechanisms partly involving the retinoid receptors [112] and has been extensively studied [113]. In *in vivo* models, fenretinide prevented the development of N-nitroso-N-methylurea-induced breast cancer in rats [114]. Recently, fenretinide has also been shown to decrease the plasma IGF-I levels in early breast cancer patients, thus suggesting its potentially chemopreventive effect [115] Indeed, in a recent large phase III trial, fenretinide use was associated with a reduction of second breast malignancies in premenopausal women with a history of previous breast cancer [116]. The rexinoid LGD 1069 was shown to be effective against established carcinogen-induced rat mammary tumors [117]. In another study, LGD 1069 prevented the development of mammary tumors in a *N*-nitroso-*N*-methylurea-induced rat mammary carcinoma model [118] and could therefore be considered as a potential agent for chemoprevention.

Non-Steroidal Anti-Inflammatory Agents - COX-2 Inhibitors

Non-steroidal anti-inflammatory drugs (NSAIDs), especially COX-2 inhibitors [66], could represent a mechanism-based chemopreventive approach for carcinogenesis. Epidemiological studies investigating the relationship between NSAID use and breast cancer have reported conflicting results; namely, some of the studies have failed to show any significant relationship [119], whereas other studies did show a relationship between NSAID use and a reduction in breast cancer by 30-40% [120]. The exact reasons for the conflict in such data are not known, but it is possible that it is partly due to the fact that only a subset of breast cancers, such as ER-negative or Her-2/neu-positive cancers express COX-2. Early studies that showed elevated levels of PG in breast tumors were the first to suggest the importance of

COX expression [121] and the levels were particularly increased in patients with metastatic disease [122]. Later, COX-2 expression was shown in breast cancer cell lines; COX-2 mRNA was especially increased in the ER-negative and highly aggressive MDA MB-231 human breast cancer cell lines [123]. Several studies have reported an increased COX-2 expression in human breast tumors [65]. The data possibly linking COX-2 to breast carcinogenesis come from a recent study demonstrating an over-expression of COX-2 from the mouse mammary tumor virus promoter to be sufficient to cause breast tumor formation in more than 85% of multiparous mice [71]. Furthermore, the selective COX-2 inhibitor, celecoxib, has been shown to be a growth inhibitory effect on breast cancer cell lines, while also inducing the regression of 7,12-dimethylbenz(*a*)anthracene-induced mammary tumors in rats [124]. Finally, the potential for COX-2 inhibitors as chemopreventive agents in breast cancer was demonstrated in several *in vivo* mouse models in which COX-2 inhibitors reduced the development of carcinogen-induced mammary tumors [125]. Currently, several phase I and phase II chemoprevention trials are planned to evaluate the COX-2 inhibitors in the context of breast cancer chemoprevention.

Lipoxygenase Inhibitors

Lipoxygenase (LOX) metabolic pathways are emerging as key regulators of cell proliferation and neo-angiogenesis. There are three known isoforms of the LOX family; 5-LOX, 12-LOX and 15-LOX. Recently, a participation of 5-LOX in the regulation of cell proliferation and apoptosis has emerged. Investigations of the biological functions of 5-LOX in cancer cells using pharmacological inhibitors and/or antisense technology have revealed that 5-LOX products, namely, 5(S)-hydroxyeicosatetraenoic acid (HETE) and leukotriene (LT) A4 but not LTB4, potently up-regulate vascular endothelial growth factor (VEGF) transcription in human malignant tumors. As VEGF is also a potent pro-angiogenic factor and is therefore crucial for tumor growth and invasion, 5-LOX may promote *in vivo* tumor development by a dual mechanism, a direct proliferative stimulus on cancer cells and a potentiation of the pro-angiogenic response by the host stromal cell. A mechanism linking COX-2 inhibition to apoptosis could lead to an increase in concentration of free, unmetabolized arachidonic acid. Disruption of the 5-LOX signaling pathway mediates growth arrest and apoptosis in breast cancer cells, and inhibits breast cancer metastases.

COX-2 and 5-LOX display similarities in expression and function in human cancer. Firstly, COX-2 and 5-LOX are co-expressed and up-regulated in quite a large number of cancer cells and human tumors, including breast cancer. Secondly, both 5-LOX and COX-2 are pro-angiogenic with a convergent targeting on VEGF expression and release. Thirdly, COX-2, as well as 5-LOX inhibitors, arrest cell cycle progression and induce apoptotic cell death in a number of cancer cells. Therefore, a combination of these agents is more effective than either agent alone and that this escape process can be attenuated by dual COX/LOX inhibition.

Other Potential Chemopreventive Agents

Several lines of investigation for improved agents are now underway. One approach is to search for SERMs that have an even more favorable profile that raloxifene, which still has thromboembolic concerns and leads to vasomotor symptoms such as hot flushes and night sweats. However, its lack of gynecologic symptoms has stimulated the search for a perfect SERM which would be anti-estrogenic for the breast, endometrium and lipid profile, but have estrogenic effects on bones and brain (vasomotor symptoms). Two compounds are in stage II human testing, arzoxifene and larzoxifene and several more are in early development. ER-negative tumors remain a challenge for prevention and new targets will be needed to prevent these tumors. There is interest in EGFR blockers [gefitinib (sp.)] and agents targeting HER2 such as trastuzamab and joint blockers of both targets (lapatinib), but these current agents are too toxic for prevention. In addition to NSAIDs [126, 127], COX-2 inhibitors [66, 128], retinoids and rexinoids [129], statins [130-132] may also protect against both ER-positive and ER-negative tumors, but only findings from observational studies or adjuvant studies or trials with other primary endpoints are available at the moment and the results still have inconsistencies.

Several other natural agents are under consideration for the chemoprevention of breast cancer, including the monoterpenes limonene and perillyl alcohol. The proposed anti-tumor and preventive mechanism includes the induction of apoptosis, cell cycle arrest and the inhibition of posttranslational modification of proteins that are involved in signal transduction and in increasing the expression of IGF-IIR [133], a putative breast tumor suppressor gene [134]. Perillyl alcohol is currently under clinical phase I testing [135]. The isoflavone, ganistein, which is found in high concentrations in soybeans and soy products, inhibits estradiol synthesis by inhibiting aromatase, inhibits DNA topoisomerase and the expression of DNA transcription factors c fos and c jun and is currently under clinical trial [136].

Signal transduction modulators such as EGFR inhibitors and farnesyltransferase inhibitors (FTIs) are also proposed as potential chemopreventive agents for breast cancer [137].

Demethylating agents are also promising agents. DNA methylation plays an important role in gene expression in breast cancers. Since epigenetic changes are potentially reversible, demethylating agents, such as 5-aza-C, can therefore possibly be utilized as potential chemopreventive agents [52]. They are especially promising for the prevention of ER-negative breast cancers, given that the demethylation of the ER gene induces the re-expression of the ER [138].

CURRENT ON-GOING PREVENTION TRIALS

Two primary prevention trials using AIs are currently in progress. One uses anastrozole while the other uses exemestane. The international breast cancer intervention (IBIS)-II trial began in February 2003 and is comparing anastrozole to placebo on 6,000 postmenopausal women at increased risk of breast cancer [139]. This study is still open to recruitment. The entry criteria are similar to IBIS-I, except that only postmenopausal women are eligible and women with mammographic density covering at least 50% of the mammogram, are also eligible. A parallel study of anastrozole *vs.* tamoxifen in 4,000 post-menopausal women with locally resected ER-positive DCIS is also being conducted as part of

this activity. Another prevention trial with AIs, MAP.3 [140], is currently underway using exemestane. This trial sponsored by the NCIC-Clinical Trials Group compares exemestane for 5 years with a placebo in 3,000 postmenopausal women at increased risk. The risk factors needed for eligibility include a Gail score > 1.66, age > 60 years, prior atypical ductal or lobular hyperplasia, or DCIS treated with a mastectomy.

CONCLUSIONS

The approaches to prevent ER-positive breast cancers have been well established and the challenge now is to reduce the side effects and find agents with very favorable benefit to risk ratios. Tamoxifen is a chemopreventive agent approved for the prevention of breast cancer in high-risk women as defined by the Gail model (>1.66) and in postmenopausal women aged 60 and older. It is available in many countries worldwide for the treatment and prevention of osteoporosis in postmenopausal women, and has also been approved in the US for reducing the risk of invasive breast cancer in postmenopausal women with osteoporosis or postmenopausal women at increased risk of invasive breast cancer. Raloxifene reduces the risk of invasive breast cancer in postmenopausal women at high risk of invasive breast cancer and in postmenopausal women with osteoporosis. In addition, it is a well established agent for the prevention and treatment of osteoporosis. There was no significant difference between raloxifene and tamoxifen in the reduction in the risk of invasive breast cancer achieved in postmenopausal women at high risk of such cancer. The choice of chemoprevention agent must consider a risk-benefit assessment for each individual patient. In this context, raloxifene is a valuable option for the prevention of invasive breast cancer in postmenopausal women with osteoporosis or at high risk of invasive breast cancer. The risk-benefit ratio in elderly women remains to be carefully assessed. Further data regarding the benefit of tamoxifen and raloxifene in genetically high-risk women such as BRCA 1 and BRCA 2 mutation carriers are forthcoming. The AIs hold promise for greater efficacy and fewer, but different side effects from SERMs. Since the benefit of tamoxifen and raloxifene in preventing invasive breast cancer was only seen in ER-positive breast cancers, there is an absolute need to develop a chemopreventive agent for ER-negative breast cancers. COX-2 inhibitors seem to be suitable candidates [68]. Other promising agents for the prevention of ER-negative breast cancers include polyamine biosynthesis inhibitors, DFMO [141], vitamin D analogs, retinoids, cyclin dependent kinase inhibitors [113], telomerase inhibitors [142], isoflavonoids [136], demethylating agents [52] and molecular chemopreventive approaches including targeted gene therapy for BRCA 1 mutation carriers [143]. The widely accepted model is to obtain pre- and post chemopreventive intervention tissue from high-risk individuals and to assess the biomodulation of specific markers as well as the intervention efficacy. In this context, ductal lavage seems to be a very promising and rather noninvasive method of collecting epithelial cells from high-risk women for analysis of morphological and molecular markers. Good biomarkers will greatly accelerate our ability to evaluate new agents and breast density is currently the most attractive candidate. However, its ability to predict the degree of risk reduction still requires validation and good serum markers are still anticipated. The prevention of ER-negative breast cancer remains an unmet challenge, but new agents offer an approach to potentially prevent these cancers as well.

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ABBREVIATIONS

ATAC	=	arimidex, tamoxifen alone or in combination
AIs	=	aromatase inhibitors
ADH	=	atypical ductal hyperplasia
STK15/BTAK	=	centrosomal serine/threonine kinase 15/breast tumor amplified kinase
CORE	=	Continuing Outcomes Relevant to Evista
COX	=	cyclooxygenase
DCIS	=	ductal carcinoma *in situ*
DH	=	ductal hyperplasia
EGF	=	epidermal growth factor
EGFR	=	epidermal growth factor receptor
ER	=	estrogen receptor
FTIs	=	farnesyltransferase inhibitors
HETE	=	5(S)-hydroxyeicosatetraenoic acid
IGF	=	insulin-like growth factor
IGF-IR	=	insulin-like growth factor receptor
IGFBP3	=	insulin-like growth factor-binding protein-3
IBIS	=	International Breast Intervention Study
LCIS	=	lobular carcinoma *in situ*
LOX	=	lipoxygenase
LT	=	leukotriene (LT)
MORE	=	Multiple Outcomes of Raloxifene Evaluation
NSAIDs	=	non-steroidal anti-inflammatory drugs
PG	=	prostaglandin
RUSH	=	Raloxifene Use for the Heart

RAR	=	retinoic acid receptor
SERMs	=	selective estrogen receptor modulators
TDLUs	=	terminal duct lobular units
VEGF	=	vascular endothelial growth factor

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Melatonin suppresses AOM/DSS-induced large bowel oncogenesis in rats

Takuji Tanaka[a,*], Yumiko Yasui[a], Mayu Tanaka[b], Takahiro Tanaka[c], Takeru Oyama[a], KM Wahidur Rahman[d]

[a] Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan
[b] Department of Pharmacy, Kinjo Gakuin University of Pharmacy, 2-17723 Omori, Moriyama-ku, Aich 463-8521, Japan
[c] Department of Physical Therapy, Kansai University of Health Sciences, 2-11-1 Wakaba, Kumatori-machi, Sennan-gun, Osaka 590-0482, Japan
[d] Department of Pathology, Karmanos Cancer Institute, Wayne State University School of Medicine, 9374 Scott Hall, 540 East Canfield, Detroit, MI 48201, USA

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ABSTRACT

The inhibitory effects of exogenous melatonin (MEL) on colon oncogenesis were investigated using an azoxymethane (AOM)/dextran sodium sulfate (DSS) rat model. Male F344 rats initiated with a single intraperitoneal injection of AOM (20 mg/kg bw) were promoted by 1% (w/v) DSS in drinking water for 7 days. They were then given 0.4, 2 or 10 ppm MEL in drinking water for 17 weeks. At week 20, the development of colonic adenocarcinoma was significantly inhibited by the administration with MEL dose-dependently. MEL exposure modulated the mitotic and apoptotic indices in the colonic adenocarcinomas that developed and lowered the immunohistochemical expression of nuclear factor kappa B, tumor necrosis factor α, interleukin-1β and STAT3 in the epithelial malignancies. These results may indicate the beneficial effects of MEL on colitis-related colon carcinogenesis and a potential application for inhibiting colorectal cancer development in the inflamed colon.

1. Introduction

The pineal production of melatonin (MEL) is controlled by an endogenous clock, the suprachiasmatic nuclei (SNC) of the hypothalamus, which is synchronized by the light/dark cycle, detected by photoreceptors located at the retinal ganglion cells. Neurons from the SCN project to the superior cervical ganglia and postganglionic noradrenergic fibers innervate pinealocytes. In darkness, the norepinephrine released from these sympathetic fibers promotes the synthesis of MEL. The maximum production of MEL within the pinealocytes occurs at night in response of a signal from the eye indication the absence of light. MEL acts as a circadian rhythm monitor, free radical scavenger and antioxidant, cytoprotective agent, immunomodulator, endocrine modulator, oncostatic agent and thermo regulator [1,2]. A pineal disturbance influences the pathogenesis and the phenotypic variations of metabolic syndrome [3]. Regarding the effects of MEL on oncogenesis, epidemiological studies provided evidence of the potential risk factor of alight at night in breast cancer with its involvement in the entire circadian axis rather than just MEL depression [4], endometrial cancer [5] and colorectal cancer (CRC) [6]. Experimental studies [7] suggest the protective effects of exogenous MEL on carcinogenesis mainly the in mammary gland [8] and other tissues including the colon [9–12], liver [13], skin [14] and pancreas [15]. The protective effects of MEL on oncogenesis are considered to be due to its antioxidative ability [16], antimutagenic potential [17] and alterations of MEL receptor-mediated metabolism [18]. Blask et al. postulated a new mechanism by which physiological and pharmacological blood levels of MEL inhibit cancer growth *in vivo* via a MEL-induced suppression of tumor linoleic acid uptake and its metabolism to the important mitogenic signaling molecule 13-hydroxyoctadecadienoic acid [19]. In addition, MEL is capable of inhibiting chemically induced colitis [20,21]. The anti-inflammatory action of MEL [22] is due to the suppression of COX-2 and iNOS expression [20] and the inhibition of nuclear factor-kappaB (NF-κB) [21]. These findings stimulate the clinical interest of MEL and suggest clinical applications of MEL and MEL agonists in oncology and chemoprevention [1].

Abbreviations: ALT, alanine aminotransferase; AOM, azoxymethane; AST, aspartate aminotransferase; COX, cyclooxygenase; CRC, colorectal cancer; DSS, dextran sodium sulfate; dUTP, deoxyuridine triphosphate; H & E, hematoxylin and eosin; HDL, high-density lipoprotein; IBD, inflammatory bowel disease; IL, interleukin; iNOS, inducible nitric oxide; LDL, low-density lipoprotein; MEL, melatonin; MI, mitotic index; NF-κB, nuclear factor-kappa B; PCNA, proliferative cell nuclear antigen; SNC, suprachiasmatic nuclei; ssDNA, single stranded DNA; T-Cho, total cholesterol; TdT, terminal deoxynucleotidyl transferase; TG, triglycerides; TNF, tumor necrosis factor; TUNEL, TdT-mediated dUTP-biotin nick end labeling; VLDL, very low-density lipoprotein.

* Corresponding author. Tel.: +81 76 218 8116; fax: +81 76 286 6926.
E-mail address: takutt@kanazawa-med.ac.jp (T. Tanaka).

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CRC is one of the leading causes of cancer deaths in the Western countries. Globally, the mortality of CRC is 655,000 deaths per year in 2005 [23]. Inflammation is known to be linked with CRC development as it is in epithelial malignancies in other tissues [24]. The risk of CRC in the patients with inflammatory bowel disease (IBD), including ulcerative colitis, increases with the increasing extent and duration of the disease [25]. A mouse model was recently established for colitis-related colon carcinogenesis [26] to investigate the pathogenesis [27–29] and chemoprevention [30,31] of inflammation-related CRC. In this mouse model of inflammation related two-stage colon carcinogenesis, different colonic carcinogens can be used in combination with a colitis-inducing agent, dextran sodium sulfate (DSS) and many colonic tumors develop within a short-term period [26,32–34]. In this model, the powerful tumor promoting effect of DSS may be due to oxidative/nitrosative stress caused by DSS-induced colitis [27–29]. This suggests that oxidative/nitrosative DNA damage associated with inflammation is involved in carcinogenesis and thus it is important to control the events that result in inflammation-related carcinogenesis [35]. In humans, oxidative stress also plays a key role in the pathogenesis of IBD-related intestinal damage [36].

Many drugs and chemopreventive agents are introduced for treatment or chemoprevention of IBD and IBD-related CRC [37]. The current study investigated whether MEL exerts cancer chemopreventive ability in colitis-associated colon carcinogenesis using a rat model [38,39], where the treatment schedule of AOM and DSS was similar to that in the mouse model [26]. In addition, the effects of MEL on the immunohistochemical expression of several biomarkers for colon oncogenesis including NF-κB, tumor necrosis factor (TNF)α, interleukin (IL)-1 and STAT3 [40,41] were studied in the colonic epithelial malignancies (adenocarcinomas). Additionally, effects of MEL on cell proliferation and apoptosis in the colonic adenocarcinomas were evaluated by the proliferation associated indices, proliferative cell nuclear antigen (PCNA) and Ki67 (MIB-5) and the apoptosis indices, the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) method and the rabbit polyclonal anti-single stranded DNA (ssDNA) method.

2. Materials and methods

2.1. Animals, chemicals and diets

Male F344 rats (Charles River Japan, Tokyo, Japan) aged 5 weeks were used in this study. The animals were maintained in Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (3 or 4 rats/cage) with free access to tap water and a pelleted basal diet (CRF-1, Oriental Yeast, Co., Ltd., Tokyo, Japan) under controlled conditions of humidity (50 ± 10%), lightning (12-h light/dark cycle) and temperature (23 ± 2 °C). They were quarantined for 7 days after arrival and randomized by body weight into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). DSS with a molecular weight of 36,000–50,000 was obtained from MP Biomedicals, LLC (Aurora, OH). DSS for induction of colitis was dissolved in distilled water at 1% (w/v). MEL (Sigma–Aldrich Chemical Co.) was dissolved in distilled water at concentrations of 0.4, 2 and 10 ppm (w/v) just before used. The preparation was done every day and animals received fresh MEL-containing drinking water in dark bottles. Animals had access to food and drinking water at all times. All handling and procedures were carried out in accordance with the Institutional Animal Care Guidelines.

2.2. Experimental procedures

The Institutional Animal Care and Use Committee evaluated all animal procedure associated with the present study and assured that all proposed methods were appropriate.

A total of 140 male F344 rats were divided into 9 experimental and control groups, as shown in Table 1. The rats in groups 1 through 6 were initiated by a single intraperitoneal injection of AOM (20 mg/kg body weight). Starting 1 week after the injection, 2% DSS in drinking water was administrated to rats of group 1 (n = 25) for 7 days and then followed without any further treatments for 18 weeks. Groups 2–4 (n = 25 for each group) were given drinking water containing 0.4, 2 and 10 ppm MEL for 17 weeks, respectively, starting 1 week after the cessation of DSS exposure. Group 5 (n = 8) received AOM and 10 ppm MEL. Group 6 (n = 8) was given AOM alone. Group 7 (n = 8) was given 2% DSS alone. Group 8 (n = 8) received 10 ppm MEL alone. Rats of group 9 (n = 8) did not receive any treatments and served as an untreated control. MEL was given to rats belonging to groups 2–5, 7 and 8 at night (from 18:00 to 9:00). The highest dose used in this experiment was based on the report by Li et al. [21], in which the dose significantly inhibited colitis-induced 2,4,6-trinitrobenzene by in rats. All animals were subjected to a complete gross necropsy examination at the time of euthanasia by CO_2 asphyxiation (week 20). The body, liver and spleen were weighed.

At necropsy, the colons were flushed with saline, excised, their length measured (from ileocecal junction to the anal verge), cut open longitudinally along the main axis and then washed with saline to remove feces. They were cut and fixed in 10% buffered formalin for at least 24 h. The histopathological examination was performed on paraffin-embedded sections, after staining with hematoxylin and eosin (H & E). Colonic tumors were diagnosed according to the Ward's description [42]. In brief, if the tumors with tubular formation invaded into the submucosa, the tumor was diagnosed as an adenocarcinoma. When the tumors with glandular structure did not invade the submucosa or depth and compressed the surrounding crypts, the tumor was diagnosed as an adenoma. The scoring (incidence and multiplicity) of the tumors was done on the H & E-stained tissue sections. The mitotic index (MI) was determined by counting number of mitoses per 100 adenocarcinoma cells on the H & E-stained sections.

2.3. Clinical chemistry

At the end of the 20-week experimental period, 5 rats randomly selected from each group were fasted overnight and then were anesthetized with sodium pentobarbital (30 mg/kg, i.p., Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) for a biochemical analysis. Blood from the inferior vena cava was collected into tubes containing EDTA and centrifuged (1500 × g, 10 min, 4 °C). The serum was aspirated and assayed as described below.

The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using commercially available kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Serum total cholesterol (T-Cho), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels were determined using commercial kits (BioVision Incorp., Mountain View, CA, USA). The serum glucose level was measured by the glucose oxidase method (Wako Pure Chemical Industries) and the serum levels of insulin (Wako Pure Chemical Industries) and leptin (GE Healthcare Bio-Sciences Corp., Picscataway, NJ, USA) were determined by sandwich ELISA kits.

Table 1
Effects of melatonin on the development of colonic adenoma and adenocarcinoma.

Group no.	Treatment	No. of rats examined	Incidence (%)		Multiplicity (no. of tumors/colon)	
			AD	ADC	AD	ADC
1	AOM/2% DSS[a]	25	100	100	7.56 ± 1.89[b]	8.16 ± 2.36
2	AOM/2% DSS + 0.4 ppm MEL	25	96	92	5.32 ± 2.19[c]	4.08 ± 2.47[d]
3	AOM/2% DSS + 2 ppm MEL	25	76[e]	72[f]	3.24 ± 2.28[d]	2.36 ± 2.43[d]
4	AOM/2% DSS + 10 ppm MEL	25	68[g]	64[h]	2.28 ± 1.99[d]	1.68 ± 2.08[d]
5	AOM + 10 ppm MEL	8	0	0	0	0
6	AOM	8	0	0	0	0
7	2% DSS	8	0	0	0	0
8	10 ppm MEL	8	0	0	0	0
9	Untreated	8	0	0	0	0

[a] AOM, azoxymethane; DSS, dextran sodium sulfate; MEL, melatonin; AD, adenoma; ADC, adenocarcinoma.
[b] Mean ± S.D.
[c] Significantly different from the AOM/DSS group (group 1) by Turkey–Kramer multiple comparison post-test ($p < 0.01$).
[d] Significantly different from the AOM/DSS group (group 1) by Turkey–Kramer multiple comparison post-test ($p < 0.001$).
[e] Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0111$).
[f] Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0048$).
[g] Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0020$).
[h] Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0008$).

2.4. Scoring of inflammation in the large bowel

Two longitudinal H & E-stained sections of a whole colon were made from all rats. Inflammation with or without mucosal ulcer in the large bowel was scored on the H & E-stained sections. For scoring, large intestinal inflammation was graded according to the following morphological criteria [43]: grade 0, normal appearance; grade 1, shortening and loss of the basal 1/3 of the actual crypts with mild inflammation in the mucosa; grade 2, loss of the basal 2/3 of the crypts with moderate inflammation in the mucosa; grade 3, loss of entire crypts with severe inflammation in the mucosa and submucosa, but retaining of the surface epithelium and grade 4, presence of mucosal ulcer with severe inflammation (infiltration of neutrophils, lymphocytes and plasma cells) in the mucosa, submucosa, muscularis propria and/or subserosa. The scoring was performed on the entire colon with or without proliferative lesions and expressed as a mean average score/rat.

2.5. Immunohistochemistry of NF-κB, TNFα, IL-1β, STAT3, PCNA, MIB-5, TUNEL and ssDNA

The immunohistochemical analysis of the colon adenocarcinoma cells for the NF-κB, TNFα, IL-1β, STAT3, PCNA, MIB-5, TUNEL and ssDNA antibodies was performed on 4-μm-thick paraffin-embedded sections by the labeled streptavidin biotin method using a LSAB KIT (DAKO Japan, Kyoto, Japan), with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65 °C, deparaffinized in xylene and rehydrated through graded ethanol at room temperature. Tris–HCl buffer (0.05 M, pH 7.6) was used to prepare the solutions and was used for washes between the various steps. Incubations were performed in a humidified chamber.

The sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4 °C with primary antibodies. The primary antibodies included anti-NF-κB p50 (H-119) rabbit polyclonal antibody (#sc-7178, 1: 500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-human TNFα rabbit polyclonal antibody (#ab6671, 1:500 dilution; Abcam, Inc., Cambridge, MA, USA), anti-mouse IL-1β rabbit polyclonal antibody (#LS-B40, 1:250 dilution; LifeSpan BioSciences, Inc., Seattle, WA, USA), anti-mouse STAT3 rabbit polyclonal antibody (#ab31370, 1:250 dilution; Abcam, Inc.), anti-human PCNA mouse monoclonal antibody (DAKO #U 7032, 1:1000 dilution; DakoCtytomation, Kyoto, Japan), mouse monoclonal antibody anti-rat Ki-67 (clone MIB-5, #M7248, 1:100 dilution; DakoCytomation) and anti-ssDNA rabbit polyclonal antibody (#A4506, 1:1000 dilution; DakoCytomation). These antibodies were applied to the sections according to the manufacturer's protocol. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, negative controls were performed on serial sections without the first antibodies.

The levels of apoptosis in tumor tissues determined by the TUNEL method were done on 4-μm formalin-fixed, paraffin-embedded tissue sections of colonic adenocarcinomas, according to manufacturer's instructions using the Apoptosis *in situ* Detection Kit Wako (Cat. No. 298-60201, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The kit is based on the TUNEL procedure. Appropriate positive and negative controls for determining the specificity of staining were generated. Negative controls were processed in the absence of the TdT enzyme in the reaction buffer. Sections of tissue digested with nuclease enzyme and colon lymphoid nodules, which are known to exhibit high rates of apoptosis, were used as positive controls. The color was developed with the peroxidase substrate 3,3′-diaminobenzidine and sections were counterstained with Mayer's hematoxylin (Merck).

2.6. Immunohistochemical evaluation

The immunoreactivity against each antibody was assessed in large colonic adenocarcinomas (more than 3 mm in diameter) developed in groups 1–4 using a microscope (Olympus BX41, Olympus Optical Co., Tokyo, Japan). Intensity and localization of immunoreactivity against the primary antibodies were determined by two pathologists (TT and TO) who were unaware of the treatment group to which the slide belonged. They evaluated the immunoreactivity against the NF-κB, TNFα, IL-1β and STAT3 antibodies with grading between 0 and 5: 0 (~15% of the colonic cancer cells showing positive reactivity), 1 (16–30% of the colonic cancer cells showing positive reactivity), 2 (31–45% of the colonic cancer cells showing positive reactivity), 3 (46–60% of the colonic cancer cells presenting positive reactivity), 4 (61–75% of the colonic cancer cells showing positive reactivity) and 5 (~75% of the colonic cancer cells showing positive reactivity).

The number of nuclei with positive reactivity for PCNA-, MIB-5, ssDNA and TUNEL-immunohistochemistry were counted in a total

of 3 × 100 cells in three different areas of the colonic cancer and expressed as percentage (mean ± S.D).

2.7. Statistical evaluation

Where applicable, data were analyzed using one-way ANOVA with Tukey–Kramer Multiple Comparisons Test or Bonferroni (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA, USA) with $p < 0.05$ as the criterion of significance. The Fisher's Exact Probability test was used for comparison of the incidence of lesions between the two groups.

3. Results

3.1. General observation

During the experiment, a few animals that received AOM/DSS (group 1) or AOM/DSS/MEL (groups 2–4) had bloody stools, but the symptom disappeared soon after stopping of the DSS treatment. At weeks 18–20, some rats of these groups had bloody stools again and anal prolapse with a rectal tumor. Other groups did not show any symptoms that were related to the treatments. The mean daily intakes of DSS (38.5 ± 1.3 ml/day for groups 1–4 and 7), MEL (9.0 ± 1.4 ml/day for groups 2–5 and 8) and distilled water (39.5 ± 0.6 ml/day for groups 6 and 9) were comparable among the groups. The rats of group 8 that received 10 ppm MEL alone for 17 weeks were healthy throughout the study. The mean body weight of group 3 (AOM/DSS/2 ppm MEL, 352 ± 11 g, $p < 0.05$) was greater than that of group 1 (AOM/DSS, 338 ± 12 g) at the end of the study. The mean liver weights did not significantly differ among the groups. The mean relative liver weight (g/100 g body weight) of group 1 (3.25 ± 0.08, $p < 0.001$) was larger than that of the untreated control group (group 9, 2.88 ± 0.05). The values of groups 2 (AOM/DSS/0.4 ppm MEL, 2.87 ± 0.04, $p < 0.001$), 3 (AOM/DSS/2 ppm MEL, 3.13 ± 0.04, $p < 0.001$) and 4 (AOM/DSS/10 ppm MEL, 3.18 ± 0.07, $p < 0.01$) were smaller than that of group 1. However, a histopathological examination revealed no significant morphological alterations in the organs other than the colon.

3.2. Pathological findings

Macroscopically, nodular and polypoid colonic tumors were observed in the middle and distal colon of rats in groups 1 through 4. These tumors were histopathologically tubular adenoma (Fig. 1A) or tubular adenocarcinoma (Fig. 1B), some of which invaded into the submucosa or serosa. Dysplastic crypts (Fig. 1C) and mucosal ulcers (Fig. 1D) were also observed in the surrounding of the neoplasms. Enlarged lymph nodes with inflammation were present around the tumors. Rats of group 5 (AOM/10 ppm MEL), group 6 (AOM alone), group 7 (2% DSS alone), group 8 (10 ppm MEL alone) and group 9 (untreated) had no tumors in all the organs examined, including the large bowel.

3.3. Incidence and multiplicity of colonic tumors and multiplicity of high-grade dysplasia

Table 1 lists the incidences and multiplicities of the colonic tumors. Group 1 (AOM/DSS) had 100% incidence of colon adenocarcinomas with a multiplicity of 8.16 ± 2.36. In contrast, the incidences of colonic adenocarcinoma of group 2 (AOM/DSS/0.4 ppm MEL, 92%), group 3 (AOM/DSS/2 ppm MEL, 72%, $p = 0.0048$) and group 4 (AOM/DSS/10 ppm MEL, 64%, $p = 0.0008$) were low in comparison to that of group 1. Also, the multiplicities of colonic adenocarcinoma of group 2 (4.08 ± 2.47, $p < 0.001$), group 3 (2.36 ± 2.43, $p < 0.001$) and group 4 (1.68 ± 2.08, $p < 0.001$) were significantly lower than that of group 1.

High-grade dysplastic crypts (Fig. 1C) developed in the large bowel of rats in groups 1 through 4 (Table 2). In comparison to group 1 (AOM/DSS), MEL treatment after AOM/DSS exposure significantly lowered the multiplicity of high-grade dysplasia.

3.4. Inflammation score in the colon

Table 2 summarizes data on scores of colonic inflammation at week 20. The mean inflammation score of group 1 (AOM/DSS, 1.88) was the greatest among the groups. The scores of group 2 (AOM/DSS/0.4 ppm MEL, 0.84), group 3 (AOM/DSS/2 ppm MEL, 0.52) and group 4 (AOM/DSS/10 ppm MEL, 0.32) were significantly lower than that of group 1. The value of group 7 (2% DSS) was less than group 1 and larger than groups 2–4. Colonic inflammation was not observed in groups 5 (AOM/10 ppm MEL), 6 (AOM), 8 (10 ppm MEL) and 9 (untreated).

3.5. Indices of mitosis, proliferation and apoptosis in colonic adenocarcinomas

The PCNA-labeling index and MIB-5-positive index of the morphologically intact colonic mucosa ($n = 5$ for each group) were 8.4 ± 1.1 and 9.4 ± 0.5 in group 1, 8.2 ± 0.8 and 8.2 ± 1.5 in group 2, 8.0 ± 0.7 and 7.6 ± 0.9 in group 3, 7.2 ± 0.4 and 7.8 ± 1.3 in group 4, 7.0 ± 0.7 and 7.4 ± 1.3 in group 5, 7.4 ± 0.9 and 7.8 ± 1.8 in group 6, 7.0 ± 1.2 and 7.4 ± 1.7 in group 7, 6.8 ± 0.8 and 7.4 ± 1.1 in group 8 and 6.6 ± 0.9 and 7.2 ± 0.8 in group 9. These values did not show a statistical significant difference among the groups. The data on the proliferative activities in the colonic adenocarcinomas determined by MI, PCNA and MIB-5 indices are illustrated in Fig. 2. As shown in Fig. 2A, the mean MIs of group 2 (AOM/DSS/0.4 ppm MEL, 25.8), group 3 (AOM/DSS/2 ppm MEL, 22.7) and group 4 (AOM/DSS/10 ppm MEL, 18.1) were smaller than group 1 (AOM/DSS, 27.3). The PCNA-labeling indices (Fig. 2B) of group 2 (AOM/DSS/0.4 ppm MEL, 65.0), group 3 (AOM/DSS/2 ppm MEL, 54.6) and group 4 (AOM/DSS/10 ppm MEL, 49.0) were significantly smaller than that of group 1 (AOM/DSS, 72.8). In addition, the MIB-5-labeling indices (Fig. 2C) of group 2 (AOM/DSS/0.4 ppm MEL, 70.3), group 3 (AOM/DSS/2 ppm MEL, 62.9) and group 4 (AOM/DSS/10 ppm MEL, 56.3) were significantly smaller than that of group 1 (AOM/DSS, 80.8). The apoptotic indices measured by TUNEL and ssDNA methods in the morphologically intact colonic mucosa ($n = 5$ for each group) were 2.8 ± 0.8 and 2.0 ± 0.4 in group 1, 3.0 ± 0.7 and 1.8 ± 0.3 in group 2, 2.8 ± 0.8 and 2.2 ± 0.4 in group 3, 2.6 ± 0.5 and 2.8 ± 0.4 in group 4, 2.8 ± 0.8 and 2.4 ± 0.5 in group 5, 6.4 ± 0.9 and 1.9 ± 0.2 in group 6, 9.0 ± 0.7 and 2.3 ± 0.4 in group 7, 2.5 ± 1.0 and 2.6 ± 0.5 in group 8 and 2.4 ± 0.8 and 2.5 ± 0.5 in group 9, respectively. The values were insignificant among the groups. As illustrated in Fig. 3, apoptotic indices determined by the TUNEL (Fig. 3A) – and ssDNA (Fig. 3B) – methods of groups 2 through 4 were significantly lower than those of group 1.

3.6. Scores of NF-κB, TNFα, IL-1β and STAT3 immunohistochemistry

The antibodies against NF-κB, TNFα, IL-1β and STAT3 were positive in the nuclei and/or cytoplasm of adenocarcinoma cells and infiltrated mononuclear cells in the stroma. Intact cryptal cells were also positive, but their intensity was very weak in comparison to that of the cancer cells. The mean scores of NF-κB, TNFα, IL-1β and STAT3 immunohistochemistry in the adenocarcinomas developed are illustrated in Fig. 4. The order of the intensity of immunoreactivity of adenocarcinoma cells was NF-κB > STAT3 > IL-1β > TNF-α.

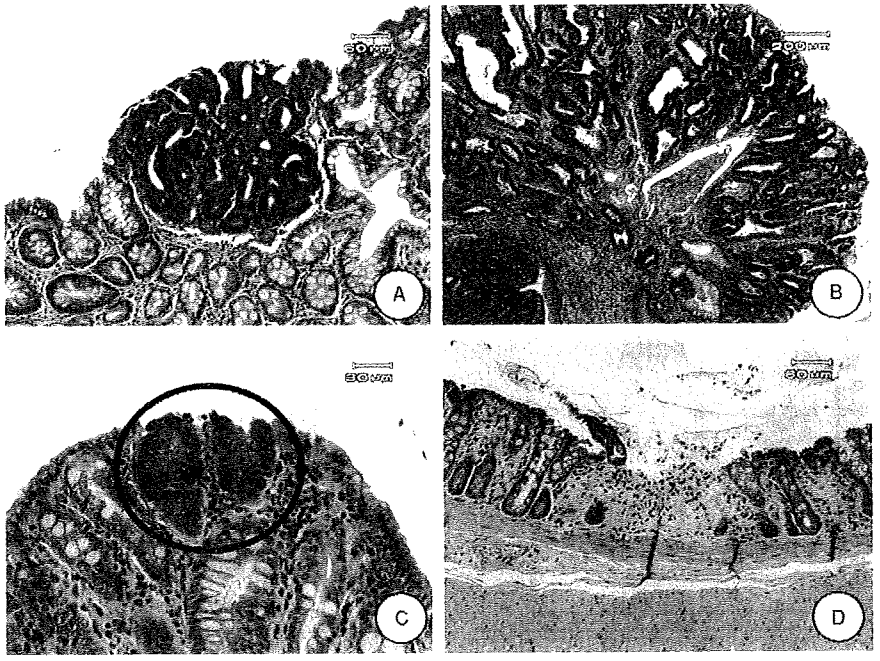

Fig. 1. Representative colonic lesions developed in rats that received AOM/DSS (group 1). (A) A tubular adenoma, (B) a well-differentiated tubular adenocarcinoma, (C) dysplastic crypts (circled) and (D) mucosal ulceration. H & E stain, bars inserted indicate magnification (A, 60 μm; B, 200 μm; C, 30 μm; D, 60 μm).

Table 2
Effects of melatonin on colonic inflammation and development of mucosal ulcer and high-grade dysplasia.

Group no.	Treatment	Number of rats examined	Inflammatory score	Number of mucosal ulcer	Number of dysplasia (high-grade)
1	AOM[a]/2% DSS	25	1.88 ± 0.78[b]	2.08 ± 0.99	11.7' ± 3.85
2	AOM/2% DSS + 0.4 ppm MEL	25	0.84 ± 0.62[c]	1.04 ± 0.68	8.08 ± 2.71[c]
3	AOM/2% DSS + 2 ppm MEL	25	0.52 ± 0.65[c]	0.60 ± 0.82[c]	6.24 ± 2.17[c]
4	AOM/2% DSS + 10 ppm MEL	25	0.32 ± 0.48[c]	0.48 ± 0.65[c]	4.72 ± 2.89[c]
5	AOM + 10 ppm MEL	8	0	0	0
6	AOM	8	0	0	0
7	2% DSS	8	1.00 ± 0.54	1.13 ± 0.64	0
8	10 ppm MEL	8	0	0	0
9	Untreated	8	0	0	0

[a] AOM, azoxymethane; DSS, dextran sodium sulfate; MEL, melatonin.
[b] Mean ± S.D.
[c] Significantly different from the AOM/DSS group (group 1) by Turkey–Kramer multiple comparison post-test ($p < 0.001$).

The mean scores of NF-κB (Fig. 4A) of group 2 (AOM/DSS/0.4 ppm MEL, 3.67), group 3 (AOM/DSS/2 ppm MEL, 2.74) and group 4 (AOM/DSS/10 ppm MEL, 1.88) were lower than that of group 1 (AOM/DSS, 4.36). The mean scores of TNF (Fig. 4B)-positivity of group 2 (AOM/DSS/0.4 ppm MEL, 3.00), group 3 (AOM/DSS/2 ppm MEL, 2.32) and group 4 (AOM/DSS/10 ppm MEL, 2.00) were lower than that of group 1 (AOM/DSS, 2.00). The mean positive scores of IL-1β (Fig. 4C) of group 2 (AOM/DSS/0.4 ppm MEL, 2.88), group 3 (AOM/DSS/2 ppm MEL, 2.53) and group 4 (AOM/DSS/10 ppm MEL, 2.12) were lower than that of group 1 (AOM/DSS, 3.92). The mean scores of STAT3 (Fig. 4D) of group 2 (AOM/DSS/0.4 ppm MEL, 2.88), group 3 (AOM/DSS/2 ppm MEL, 2.47) and group 4 (AOM/DSS/10 ppm MEL, 2.31) were lower than that of group 1 (AOM/DSS, 4.24).

3.7. Clinical chemistry of serum levels of AST, ALT, T-Cho, TG, glucose, leptin, insulin, VLDL, LDL and HDL

The data on the clinical chemistry of the serum levels of AST, ALT, glucose, leptin and insulin are shown in Fig. 5 and those of T-Cho, TG, VLDL, LDL and HDL in Fig. 6. The administration of MEL dose-dependently lowered the levels of AST (Fig. 5A), ALT (Fig. 5B), insulin (Fig. 5E), T-Cho (Fig. 6A), TG (Fig. 6B), VLDL (Fig. 6C) and LDL (Fig. 6D) when in comparison with that of group 1, but the differences were insignificant. In contrast, MEL exposure at dose levels of 2 and 10 ppm significantly lowered serum levels of glucose (Fig. 5C) and leptin (Fig. 5D). MEL at a dose of 10 ppm significantly elevated the HDL level (Fig. 6E).

4. Discussion

The results presented herein clearly indicated that MEL in drinking water effectively suppresses AOM/DSS-induced rat colitis-related colonic oncogenesis without any adverse effects. The administration of MEL exerted a cancer chemopreventive ability by suppressing several biomarkers [40,41] of colon carcinogenesis. This is the first report showing that prolonged dosing of exogenous MEL exerts cancer chemopreventive ability in colitis-related colon carcinogenesis in rodents. Although other treatment methods of MEL have been previously reported [44], MEL was given in drinking water to rats in this study, as in Sener et al. [45] in order to obtain an effective biological activity of MEL throughout the experiment.

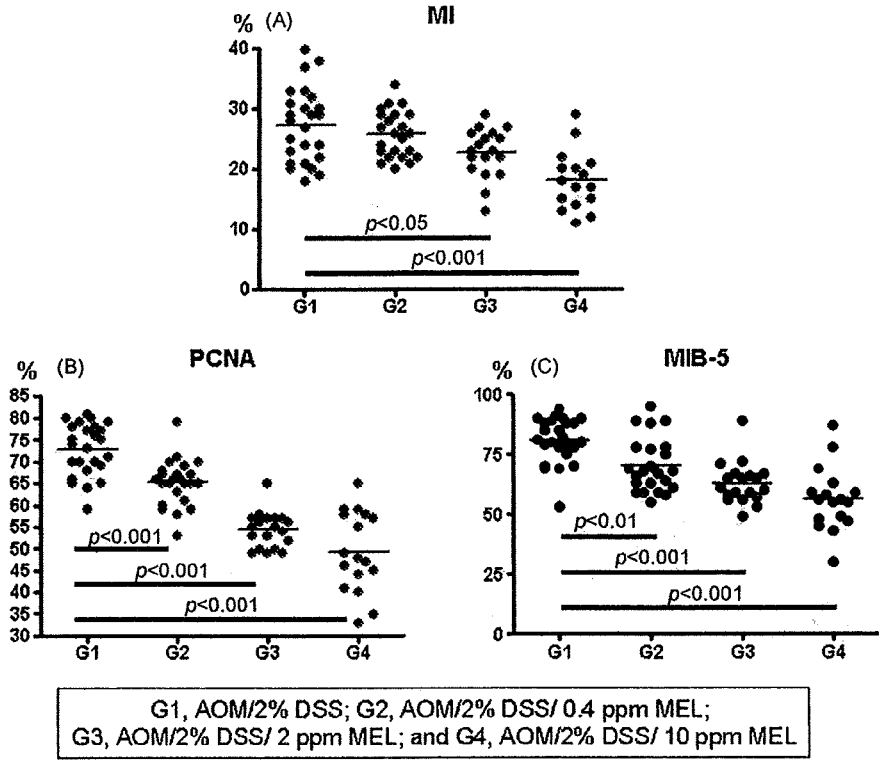

Fig. 2. The mitotic index (MI, panel A) and proliferation indices (PCNA, panel B; MIB-5, panel C) of the adenocarcinomas developed in groups 1 through 4.

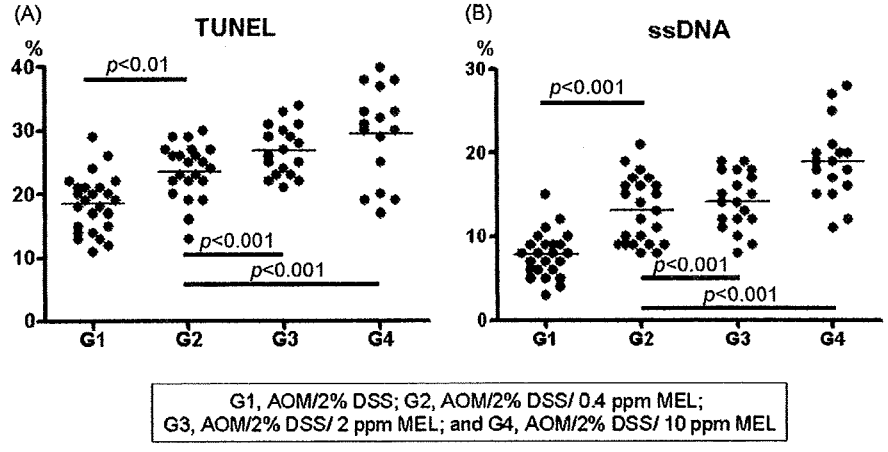

Fig. 3. The apoptotic indices (TUNEL, panel A; ssDNA, panel B) of the adenocarcinomas developed in rats of groups 1 through 4.

Importantly, the 17 weeks administration of MEL (group 8) did not cause any toxic effects in the rats. In this study, the highest dose (10 ppm) of MEL we selected, since that dose inhibits chemically induced colitis in rats [21]. In addition, continuous low doses (0.4, 2 and 10 ppm) of MEL were administered to rats, considering the fact that serum MEL level of adults is about 17 pg/ml at night and the half-life of MEL is about 30 min.

As reported previously in the experiment using mice [26], the incidence and multiplicity of colonic tumors in the rats that received AOM and 2% DSS in the current study were quite high, suggesting that a rat model using AOM and DSS can be utilized to investigate the pathogenesis of colitis-related colon carcinogenesis. The findings presented here also strengthened the importance of inflammation in colonic oncogenesis. In this study, MEL exposure was capable of inhibiting the incidence and multiplicity of colonic epithelial neoplasms (adenoma and adenocarcinoma) together with the frequency of dysplastic crypts in a dose-dependent manner. These findings are important, since the findings that the suppressing effects of MEL on the development of both preneoplasia and neoplasia in the colon suggest the possible application of MEL for clinical use in people at high-risk peoples for CRC.

In the current study, the treatment with MEL in drinking water significantly lowered colonic inflammation induced by DSS. Chronic inflammation is involved in oncogenesis in certain tissues including the large bowel, thus, suppression of chronic inflammation through modulation of expression of several pro-inflammatory gene products that mediate several events of carcinogenesis may result in cancer chemoprevention [46]. MEL has anti-inflammatory

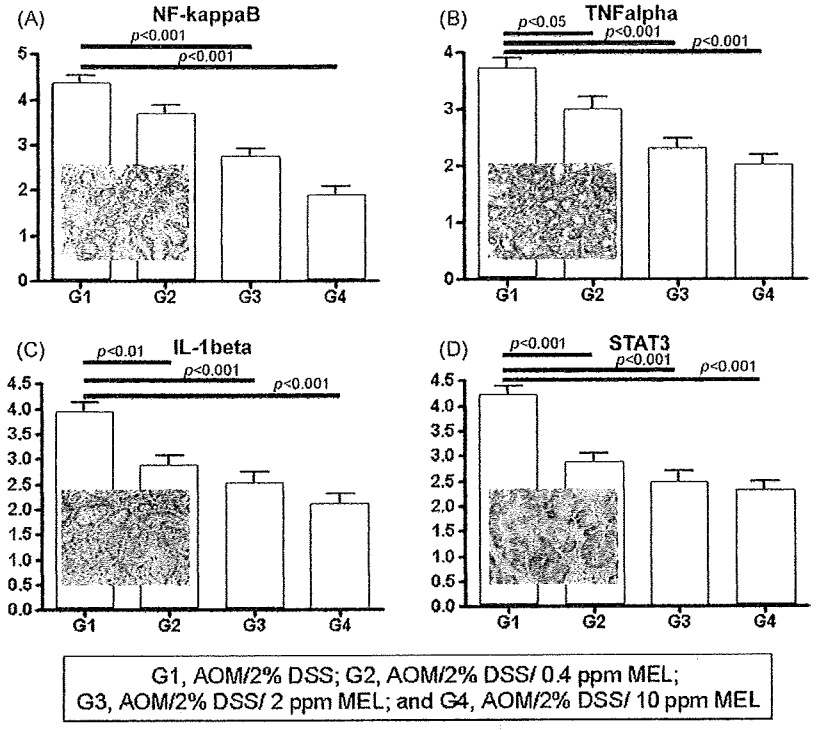

Fig. 4. The immunohistochemical scores of NF-κB (panel A), TNFα (panel B), IL-1β (panel C) and STAT3 (panel D) of the adenocarcinomas developed in rats of groups 1 through 4. Representative immunoreactivity against these antibodies in the cancer cells are inserted. Bars are 30 μm.

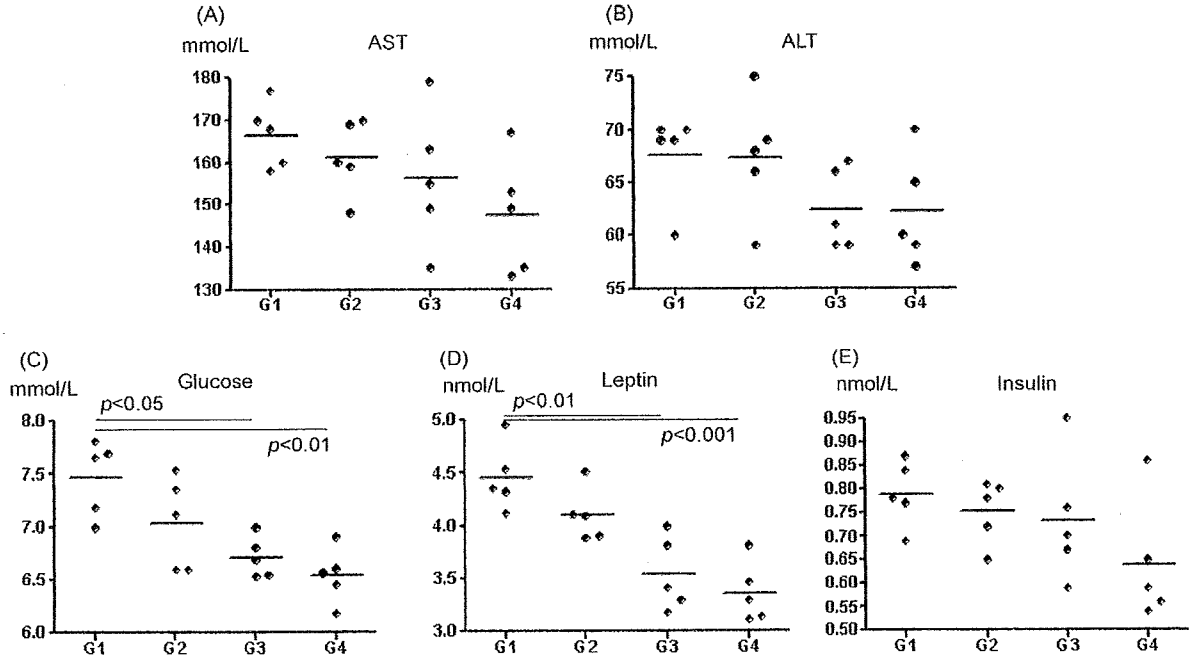

Fig. 5. Serum levels of AST (panel A), ALT (panel B), glucose (panel C), leptin (panel D) and insulin (panel E) of rats in groups 1 through 4.

effects and inhibits iNOS and COX-2 expression [20,47]. The modulation of inflammation and expression of COX-2 and iNOS in the colon results in the suppression of colitis-related colon carcinogenesis of mice [48]. Since several molecular targets for suppression of inflammation-associated carcinogenesis are proposed [49], further studies are warranted to determine the detailed mechanisms by which MEL inhibits inflammation-related carcinogenesis. In this study, MEL treatment modified the expression of NF-κB, TNFα, IL-1β and STAT3. There are candidate biomarkers of colon tumorigenesis [40,41], since the expression of NF-κB, TNFα and IL-1β is involved in colonic tumorigenesis by affecting proliferation and apoptosis [50–53]. In addition, STAT3 expression is an important factor in colon carcinogenesis, tumor invasion [54] and survival/proliferation of colonic preneoplastic cells [55]. In addition,

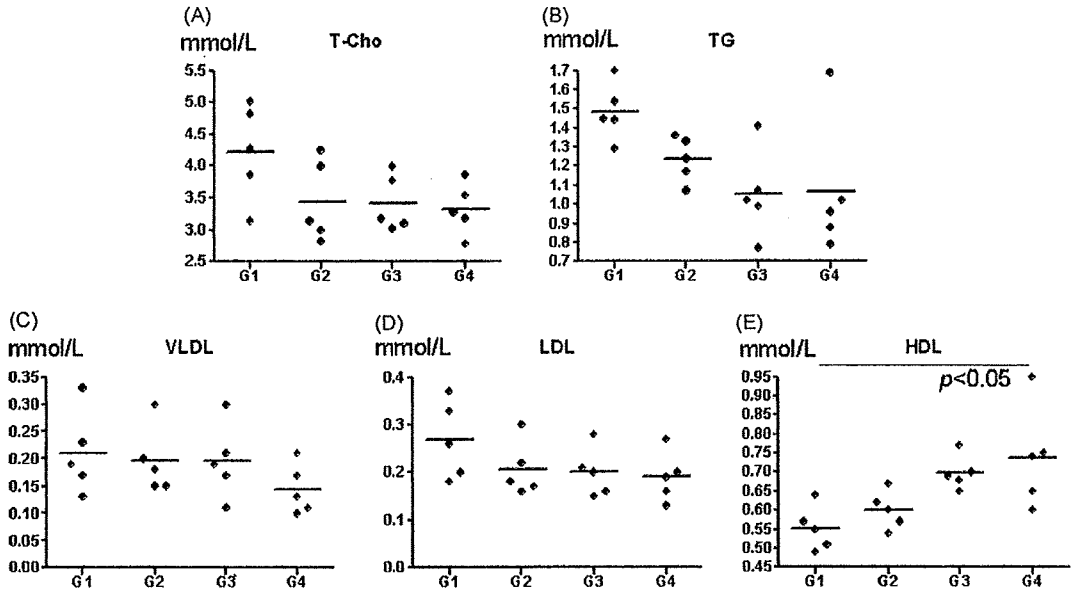

Fig. 6. Serum levels of T-Cho (panel A), TG (panel B), VLDL (panel C), LDL (panel D) and HDL (panel E) of rats in groups 1 through 4.

the anti-inflammatory potential of melatonin through suppression of the expression of NF-κB and chemokines (interleukin-8 and monocyte chemoattractant protein) in a rat colitis model [21,56] is of interest and important for further investigation of cancer chemopreventive ability of this bioactive substance, as observed in this study.

The current study measured the effects of exogenous MEL on the liver function (AST, ALT), lipid profile (T-Cho, TG, VLDL, LDL and HDL) and metabolic alteration (glucose, leptin and insulin). All measurements were the greatest in group 1 that received AOM/DSS, as observed in previous experiments using mice (unpublished work). MEL exposure lowered the serum levels of AST and ALT that were elevated by the treatment with AOM/DSS. Although the differences were insignificant, the findings may suggest the hepatoprotective effects of MEL [57]. In addition, MEL administration influenced the lipid profile. Although the effects on the serum levels of T-Cho, TG, VLDL and LDL were insignificant, an elevation of serum HDL level was observed, as reported by others [45,58]. In addition, MEL lowered the serum levels of glucose and leptin [59,60], thus suggesting the possible application of MEL in the management of obesity and/or diabetes [61].

In conclusion, prolonged exogenous MEL in drinking water was thus found to effectively inhibit colonic cancer development in a two-stage colitis-related rat colon oncogenesis model through modulation of inflammation and proliferation in the inflamed colon of rats that received AOM and DSS. The inhibition by MEL might be mediated by modulating the expression of NF-κB, TNFα, IL-1β and STAT3.

Conflict of interest

None declared.

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