Melatonin and Retinyl Acetate as Chemopreventives in DMBA-Induced Mammary Carcinogenesis in Female Sprague-Dawley Rats

(rat mammary carcinogenesis / melatonin / retinyl acetate)

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Abstract. One goal of experimental oncology is to find and test effective chemopreventive substances which can suppress malignant transformation of the cells, their accumulation and invasion. Mammary gland tumours were induced by DMBA applied intragastrically (10 mg/rat, three times) every three days between postnatal days 50 and 60 in female Sprague-Dawley rats. One day after the last dose we started chemoprevention with Mel, RA and combination of both drugs, which lasted 25 weeks. Mel was drunk continuously as a solution in tap water (100 μg/ml). RA was applied daily in the dose of 8.2 mg/rat at the base of the tongue. There were four experimental groups: 1. control – no chemoprevention, 2. Mel treatment, 3. RA treatment, 4. application RA+Mel. At the end of the experiment the incidence, frequency, latency and average volume of tumours were evaluated. In the group treated with Mel tumour incidence, latency and volume did not differ from controls; the frequency of tumours was decreased. Treatment with RA and with combination RA+Mel decreased mammary tumour incidence to 38% (RA) and 48% (RA+Mel); it also decreased frequency and prolonged latency. Thus chemoprotective effects of RA and combination of RA with Mel were proved in mammary carcinogenesis induced by DMBA. The oncostatic effect of Mel alone was not confirmed. In our recent paper (Bojková et al., 2000) drinking of lower doses of Mel during the late afternoon and night prolonged the latency period and in combination with RA showed an oncostatic effect on mammary carcinogenesis induced by NMU. Further studies are needed to elucidate the conditions of successful chemoprevention with Mel.

Breast cancer in women is a serious problem of clinical medicine and represents a demanding enigma for experimental oncology, which studies the effectiveness of chemopreventive substances, principally natural components of nutrition, and substances preventing the origin and accumulation of malignant cells by support of physiological mechanisms (Noguchi et al., 1996). A number of chemopreventive substances were tested in experimental animals against mammary gland carcinomas induced by chemocarcinogens, mostly by 7,12-dimethylbenz(a)anthracene (DMBA) and N-methyl-N-nitrosourea (NMU) in order to use them for possible application in treatment strategy in humans (El-Bayoumy, 1994). Recently, retinoids – natural and synthetic derivates of vitamin A - are besides of various types of antioestrogens most frequently used in clinical chemopreventive trials (for review see Gerster, 1993; Coradini et al., 1997; Evans and Kaye, 1999). For the first time, the anticarcinogenic effect of retinoids has been shown by Moon et al. (1976) in mammary carcinoma induced by DMBA in female Sprague-Dawley rats. Model experiments *in vitro* and *in vivo* demonstrated that retinoids and their derivates recover the balance of cellular processes disturbed by chemocarcinogens through intervention to the cell cycle, where they produce an antiproliferative effect via stimulation of maturation and differentiation of cells through induction of transforming growth factor-ß activity (TGF-ß). Retinoids increase the intercellular communication in the gap junction region by induction of connexin 43 synthesis, they adapt the tumour-suppressor function of p53, inhibit the synthesis of prostaglandins and also block the process of tumour angiogenesis (El-Bayoumy, 1994; Kelloff et al., 1997). Induction of apoptosis by increasing TGF-ß synthesis and inhibiting the telomerase belong to further oncostatic properties of retinoids (Kelloff et al., 1997). The disappearance of expression of the nuclear receptor for retinoic acid was recorded in tumour tissue of women with mammary carcinoma (Widschwendter et al., 1997).

The question about the effect of melatonin (Mel) in chemoprevention of carcinogenesis remains open. Mel, the main hormone of the pineal gland in vertebrates, is synthesized in circadian rhythm (high concentration in the dark and low concentration in the light), which is the principle of its function as a synchroniser of biological rhythms. It has antigonadotrophic, immunostimulative,

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Abbreviations: DMBA – 7,12-dimethylbenz(a)anthracene, 4-HPR – N-(4-hydroxyphenyl)retinamide, NMU – N-methyl-N-nitrosourea, Mel – melatonin, RA – retinyl acetate.

antioxidative and oncostatic effects in hormone-dependent tumours, mainly (for review see Arendt, 1995; Reiter, 1995). In pinealectomized animals increased growth of mammary tumours was found and application of Mel inhibited the growth of mammary gland tumours induced in female rats by DMBA (Tamarkin et al., 1981), resp. NMU (Blask et al., 1991). The time of the day was important for the oncostatic properties of Mel, the end of light and darkness being the most effective period; the effectiveness of Mel was dose-dependent (Hill and Blask, 1988; Subramanian and Kothari, 1991). Mel inhibited the growth of human mammary cell line MCF-7 in physiological concentrations (10^{-9} up to 10^{-11} M), higher and lower doses were not effective (Hill and Blask, 1988).

We used Mel alone or in combination with retinyl acetate (RA) and RA alone in chemoprevention of mammary carcinogenesis induced by DMBA. We supposed that the antiproliferative effect of retinoids on epithelial cells in the mammary gland, their supporting effect on the tumour-suppressor function of p53 connected with antigonadotrophic (against mitogenic-active hormones), antioxidative and immunostimulative activities of melatonin could be an effective oncostatic combination.

Material and Methods

Female Sprague-Dawley rats (AnLab, Prague, Czech Republic) were adapted to standard vivarium conditions (t=23 ± 2°C, relative humidity 60–70%) and artificial regimen (light : dark = 12 : 12 h). Cold light (fluorescent lamps Tesla, 40W), with an intensity of 150 lux in the cage, lit on automatically at 7 a.m. Rats were fed with a standard laboratory diet PM (Top-Dovo, Dobrá Voda, Slovakia) and tap water ad libitum, four animals were housed in each cage. Freshly prepared DMBA (Sigma, Deisenhofen, Germany) dissolved in flax oil was applied to all experimental groups (10 mg/rat, three times) every three days between postnatal days 50 and 60, intragastrically. After the last dose of DMBA we started with administration of chemopreventive substances, Mel, RA, and combination RA+Mel, daily during 25 weeks (June 1997 – January 1998). Mel (Biosynth, Staad, Switzerland) was drunk in tap water continuously at the concentration of 100 μg/ml of water. The stock solution was prepared by dissolving 100 mg of Mel in 2 ml of ethanol and tap water was added up to 1000 ml. The solution was changed each third day. RA (Fluka, Buchs, Switzerland) was applied at the base of the tongue by means of a pipette in the dose of 8.2 mg/rat/day, represented by 20 μg of oil concentrate, 1 mg of oil concentrate containing 1500 I.U. of pure RA.

There were four experimental groups in our study: (1) rats treated with DMBA, without chemopreventive substances (n=16), (2) rats treated with DMBA+Mel (n=10), (3) rats treated with DMBA+RA (n=16), (4) rats treated with DMBA+RA+Mel (n=26). All animals were weekly weighed and palpated, and the presence, localization, number and size of tumours were recorded. After the 25th week, animals were killed by fast decapitation, tumours were excised, weighed, measured, and fixed in formol for histological examination. Statistical significance of the differences in incidence (% of animals in the group with at least one mammary gland tumour) was calculated by χ^2 test ($P < 0.05$, $P < 0.01$), and that of latency (time period up to the detection of the first tumour) and frequency (average number of tumours per one tumour-bearing animal) by the Kruskal-Wallis test. The average tumour volume was calculated according to the formula $V = \pi.(S_1)^2.S_2/12$ (S_1 and S_2 are the tumour diameters, $S_1 < S_2$) and evaluated by analysis of variance by Kruskal-Wallis ($P < 0.05$, $P < 0.01$).

Results

Final values of incidence, latency, frequency and tumour volume are shown in Table 1. Application of Mel did not change significantly the latency period, incidence and mean tumour volume, but decreased the frequency of tumours in comparison with the control group. Retinyl acetate alone and in combination with Mel significantly decreased the incidency (to 38% RA, to 48% RA+Mel), tumour frequency and prolonged the latency. The decrease in the mean tumour volume in groups treated by administration of RA or Mel, respectively, was not statistically significant, the application of RA+Mel even increased the tumour volume in comparison with controls. Intergroup differences in body weight were not found.

Table 1. Effects of melatonin and retinyl acetate on DMBA-induced mammary tumour incidence, latency, frequency and volume in female Sprague-Dawley rats

Groups	Number of animals	Incidence (%)	Latency period (days)	Frequency (per animal)	Average tumour volume (cm^3)
DMBA (controls)	16	83	72.00 ± 6.88	7.1	4.36 ± 1.99
DMBA+Mel	10	100	97.25 ± 12.80	3.3*	1.73 ± 0.50
DMBA+RA	16	38**	110.17 ± 15.10*	2.2*	1.24 ± 0.71
DMBA+RA+Mel	26	48*	104.38 ± 8.68**	1.8*	6.26 ± 1.60*

Values of tumour latency and volume are means ± S.E.M. Statistical significance of the differences between the values of control vs. the respective group treated with chemopreventive substance(s): *$P < 0.05$, **$P < 0.01$.

Discussion

The goal of this paper was to prove the oncostatic effect of Mel and RA, administered alone or in combination, on mammary carcinogenesis induced by DMBA in female rats of the Sprague-Dawley strain. Our experiments confirmed a reliable oncostatic effect of RA, which significantly decreased the incidence of tumours by 45%, as well as their frequency, after 25-week application. We have achieved a similar anticarcinogenic effect of RA as has been published for experimental tumours of the mammary gland induced by DMBA (Moon et al., 1976) or NMU (Moon et al., 1977). RA and N-(4-hydroxyphenyl)retinamide (4-HPR) applied in food were the most efficient of all tested retinoids in reducing the incidence and frequency and prolonging the latency period in mammary gland tumours induced by NMU in rats. Both retinoids had an antiproliferative effect on the epithelium in the ductal system and in the end buds of the mammary gland (Moon et al., 1992). Contrary to other presented results and other above cited papers, no chemopreventive effect of RA was noted in NMU-induced mammary carcinogenesis in other experiments of our working group. The female Sprague-Dawley rats were used, the experimental conditions, the dose and administration of RA were the same, but the application of RA began before NMU administration (Bojková et al., 2000). Hill and Grubbs (1992) described similar chemopreventive efficacy of RA in rat mammary gland cancer induced by differently acting carcinogens: NMU and DMBA.

No chemopreventive effect of Mel was found in this experiment, but a significant decrease in the tumour frequency was recorded. Continuous application of Mel in tap water did not influence the tumour incidence. Our paper did not confirm the anticarcinogenic effect of Mel, as has been described for example by Kothari (1987) in female rats of the Holtzman strain which drank Mel in the period of tumour initiation by DMBA. The decrease in mammary tumour incidence after daily injection of Mel (500 μg/rat) at the end of the day in the period of carcinogenesis initiation induced by NMU in female rats of Sprague-Dawley strain was also recorded by Blask et al. (1991). Blask et al. (1999) investigated the inhibiting effect of Mel on tumour growth from the level of organism to the molecular level in the rat hepatoma model. The authors confirmed that the oncostatic effects of Mel are indeed circadian time-dependent. In our above cited paper (Bojková et al., 2000) Mel given in the dose of 20 μg/ml of tap water only during the late afternoon and night was more effective in chemoprevention of rat mammary tumours induced with NMU than in our present experiment. The antiproliferative effect of Mel was proved in MCF-7 cell cultures, where the cell cycle was slowed down during the G0/G1 transition to the S phase (Blask et al., 1992). Mel blocked the effect of prolactin and growth factors, transforming growth factor (TGF) and epidermal growth factor (EGF), which stimulate the growth of the tumour cell line MCF-7. It is therefore possible to suppose that Mel influenced the signal transduction or gene expression of tumour cells (Blask et al., 1997). Mel decreased the invasion of MCF-7 cells through the basal membrane by increasing integrin ß1 subunit and E-cadherin expression, and it also increased the differentiation of tumour cells (Cos et al., 1998). The oncostatic effect of Mel in experimental mammary carcinogenesis was not unambiguously confirmed in some *in vitro* (Baldwin and Barret, 1998, Papazisis et al., 1998) and *in vivo* experiments (Bartsch and Bartsch, 1994). Mel used in the adjuvant therapy of patients with advanced cancer, where standard therapy had failed, often improved the status of the patient (Panzer and Viljoen, 1997).

The reasons for Mel failure in our experiment might be different. We take into account too high doses of Mel (100 μg/ml of water, daily intake 20–30 ml of the water solution per animal) and its continuous administration. Further investigation is required to elucidate the irregular and unstable effect of Mel as a chemopreventive substance in experimental mammary carcinogenesis.

Combination of chemopreventive substances displaying different mechanisms of effect can increase their oncostatic effectiveness. Rao et al. (1990) used the combination of six substances (RA, tamoxifen, tocopherol, sodium salt of selenium, ergocryptine and aminoglutethimide) to decrease the incidence of mammary gland tumours induced by DMBA. The incidence of tumours in the control group (62%) decreased consecutively in dependence on the number of substances applied in combination up to 8.3%, when all modulators were used. The anticarcinogenic effect of retinoids, namely 4-HPR, to mammary gland carcinoma induced by NMU was increased by combination with various substances, chiefly with antioestrogen tamoxifen, and manifested itself by reduction of tumour incidence (Moon et al., 1992). New data and perspectives were presented by Coradini et al. (1997), where application of 4-HPR with tamoxifen and interferon-ß had an additive/synergic effect on suppression of the growth of oestrogen-positive (MCF-7, T47D) and oestrogen-negative (MDA-MB and BT20) mammary gland tumour cell lines.

Combination of RA and Mel in our experiment significantly prolonged latency, reduced frequency of tumours and significantly, but at a lower probability level than application of RA alone, reduced tumour incidence. Surprising was the increased average tumour volume in this group. In combined application of RA and Mel, anticarcinogenic effect greater than that caused by separate application of RA was not recorded, which indicates RA as the main oncostatic drug in this group. In our above cited paper (Bojková et al., 2000) RA and Mel given separately did not show nearly any oncostatic effect, but the combination of both substances was successful. We assumed that the initiating effect of the chosen

chemocarcinogen is of substantial importance. We have found no information about combined administration of RA and Mel in chemoprevention of experimental mammary carcinogenesis and our results represent the first report in this field.

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