

Significant Differences in the Effects of Magnetic Field Exposure on 7,12-Dimethylbenz(*a*)anthracene-Induced Mammary Carcinogenesis in Two Substrains of Sprague-Dawley Rats

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ABSTRACT

We have shown previously (S. Thun-Battersby *et al.*, *Cancer Res.*, 59: 3627–3633, 1999) that power-line frequency (50-Hz) magnetic fields (MFs) at μ T-flux densities enhance mammary gland tumor development and growth in the 7,12-dimethylbenz(*a*)anthracene (DMBA) model of breast cancer in female Sprague-Dawley (SD) rats. We also demonstrated that MF exposure results in an enhanced proliferative activity of the mammary epithelium of SD rats (M. Fedrowitz *et al.*, *Cancer Res.*, 62: 1356–1363, 2002), which is a likely explanation for the cocarcinogenic or tumor-promoting effects of MF exposure in the DMBA model. However, in contrast with our data, in a similar study conducted by Battelle in the United States, no evidence for a cocarcinogenic or tumor-promoting effect of MF exposure was found in the DMBA model in SD rats (L. E. Anderson *et al.*, *Carcinogenesis*, 20: 1615–1620, 1999). Probably the most important difference between our and the Battelle studies was the use of different substrains of SD rats; the United States rats were much more susceptible to DMBA than the rats used in our studies. This prompted us to compare different substrains of SD outbred rats in our laboratory in respect to MF effects on cell proliferation in the mammary gland, susceptibility to DMBA-induced mammary cancer, and MF effects on mammary tumor development and growth in the DMBA model. The SD substrain (termed “SD1”) used in all of our previous studies was considered MF-sensitive and used for comparison with another substrain (“SD2”) obtained from the same breeder. In contrast with SD1 rats, no enhanced cell proliferation was determined after MF exposure in SD2 rats. MF exposure significantly increased mammary tumor development and growth in SD1 but not SD2 rats. These data indicate that the genetic background plays a pivotal role in effects of MF exposure. Different strains or substrains of rats may serve to evaluate the genetic factors underlying sensitivity to cocarcinogenic or tumor-promoting effects of MF exposure.

INTRODUCTION

The generation, transmission, and use of electric energy is associated with the production of weak electric and magnetic fields which oscillate 50 or 60 times/second (power-line frequency). Over the last 2 decades there has been growing concern about whether residential or occupational exposure to power-line frequency magnetic fields (MFs) might be adverse to the human health (1–5). On the basis of epidemiological studies of childhood leukemia, it was concluded that 50/60-Hz MFs are possibly carcinogenic to humans (3, 5). Several epidemiological studies also indicated that occupational or residential MF exposure may be a risk factor for breast cancer (6–8). A plausible biological mechanism to account for such findings is the electric power/breast cancer hypothesis, also known as “melatonin hypothe-

sis” (9). This hypothesis holds that because MFs can decrease the production and release of melatonin by the pineal gland, there is an increased proliferation of breast epithelial stem cells at risk of malignant transformation (9). In line with this hypothesis, we found previously that prolonged exposure of female Sprague-Dawley (SD) rats to 50-Hz MFs at flux densities in the μ Tesla (μ T) range increases cell proliferation in the mammary gland (10), and enhances mammary tumor development and growth in response to the chemical carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA; Refs. 11–13).

Our data prompted the United States National Toxicology Program to initiate MF studies that were an attempt to replicate the results obtained by our group using the DMBA initiation/promotion mammary gland tumor model. The National Toxicology Program studies were conducted by Anderson *et al.* (14) at Battelle (Richland, WA). In contrast to our data, the Battelle studies found no evidence for a cocarcinogenic or tumor-promoting effect of MF exposure (14–16). The investigators from the two groups recently discussed differences between their studies that might explain the apparent discrepancies between the results of MF exposure (17). Probably the most important difference was the use of different substrains of SD outbred rats; the United States rats were much more susceptible to DMBA but possibly less sensitive to MF than the European rats used in our studies. It has been demonstrated previously that there are inherent differences between substrains of SD rats obtained in the United States and Europe in regard to their mammary neoplastic response to DMBA, as well as in their response to radiation (18).

This prompted us to directly compare different substrains of SD outbred rats in our laboratory with respect to MF effects on cell proliferation in the mammary gland, susceptibility to DMBA-induced mammary cancer, and MF effects on mammary tumor development and growth in the DMBA model. The SD substrain (termed “SD1”) used in all of our previous studies was considered MF-sensitive and used for comparison with other substrains. By using the activity of ornithine decarboxylase (ODC) in mammary tissue as a sensitive indicator of the effects of MF on cell proliferation (19, 20), we recently found a SD substrain (termed “SD2”) that is insensitive to the ODC-enhancing effect of MF exposure. In the present study, we compared the effects of MF exposure on ODC activity and proliferation of the mammary epithelium in SD2 rats, using *in vivo* labeling of proliferating cells with bromodeoxyuridine (BrdUrd) and *in situ* labeling of the nuclear proliferation-associated Ki-67 protein by the antibody MIB-5. Furthermore, the susceptibility of the SD1 and SD2 substrains to DMBA and to the cocarcinogenic or tumor-promoting effect of MF exposure in the DMBA model was compared.

MATERIALS AND METHODS

Animals. Female SD outbred rats (CD) were obtained from Charles River (Sulzfeld, Germany). The SD1 rats used in all of our previous MF experiments were from another breeding area (area 12) than the SD2 rats (area 3). SD2 rats were considered by the breeder to be genetically different from the SD1 substrain, because the two breeding colonies had a different history, were

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maintained strictly separated from each other over many years, and differed in the sources of SD rats used for in-migration.

For the present experiments, all of the rats were obtained at an age of 42–44 days and were acclimatized for 7–10 days in one of the animal rooms in the Department of Pharmacology before being used for the MF experiment. The experimental protocols used in this study were in line with national and international ethical guidelines, and were conducted in compliance with the German Animal Welfare Act and approved by the responsible governmental agency, including approval by an animal ethics committee. All efforts were made to minimize pain or discomfort of animals used.

ODC Activity and Cell Proliferation. Groups of 12 SD2 rats were MF- or sham-exposed for 2 weeks (for exposure details see below). In the morning of the last day of exposure, all of the rats received an i.p. injection of BrdUrd (50 mg/kg). Three h after administration of BrdUrd, all of the rats were killed by cervical dislocation. For preparation of the mammary glands, the skin was opened by a midline incision to expose the six pairs of mammary glands extending from the salivary glands to the perianal region. Specific mammary glands were identified by site as L(left)1 through L6 and R(right)1 through R6, with 1 being the most cranial and 6 the most caudal gland. Because we found previously that the sensitivity of the six mammary gland complexes to MF exposure differs with highest sensitivity in the thoracic glands (12, 17, 19, 20), BrdUrd and Ki-67 labeling was performed in the left cranial thoracic (L1) glands. Because the mammary tissue is tightly bound to the dermis, mammary tissue was always excised together with the adjacent skin, which was shaved immediately before tissue sampling. Preparation of the samples was done on a metal plate (which was cooled by dry ice), and samples were stored at -80°C until immunohistochemistry. The protocols for *in vivo* BrdUrd incorporation and subsequent immunohistochemical labeling in proliferating cells and for *in situ* Ki-67 immunolabeling by the antibody MIB-5 were based on previous experiments of Westermann *et al.* (21) and Luetig *et al.* (22), and adapted to the mammary gland as described in detail recently (10). The percentage of BrdUrd- and Ki-67-positive cells, *i.e.*, the labeling index, in sections of mammary gland tissue was determined by a person who was blind to the conditions of the section (*i.e.*, whether the section was from a MF-exposed or sham-exposed rat). An average of about 600–900 cells was counted for each section stained for detection of either BrdUrd- or Ki67-positive cells. On the basis of the quality of the immunostaining, sections from 20 rats could be finally evaluated for BrdUrd staining and sections from 18 rats for MIB staining.

For determination of ODC activity in mammary tissue, the right cranial thoracic (R1) glands from the same rats were used. Immediately after sampling, tissue specimens for the measurement of ODC activity were stored at -80°C until analysis. Storage time was kept as short as possible (maximum 1 day) to minimize the possibility of alterations in ODC activity during storage. Methods used for tissue preparation and for determination of ODC activity are described in detail elsewhere (20) and were the same as used previously for SD1 rats. The amount of ODC in the samples was measured by the method of Beaven *et al.* (23) as the amount of $^{14}\text{CO}_2$ evolved from L-[1- ^{14}C]L-ornithine (50–62 mCi/mmol; Amersham, Braunschweig, Germany) during a 180-min incubation at 37°C at a total concentration of unlabeled L-ornithine of 0.2 mM. The amount of released $^{14}\text{CO}_2$, which is correlated to the ODC activity, was related to the protein content of the samples. For additional details see Mevissen *et al.* (20).

We did not perform parallel proliferation experiments with MF exposure in SD1 rats, because previous experiments in various groups of SD1 rats have shown that MF exposure (50-Hz, 100 μT , for 2 weeks) significantly increases both ODC, and BrdUrd and Ki-67 labeling in the mammary epithelium, particularly in the thoracic glands, in this SD substrain (10, 20).

DMBA Treatment. Two experiments were performed with DMBA. In a first experiment (“experiment 1”), the two substrains (20 rats/substrain) were compared in their carcinogenic response to DMBA in the absence of MF exposure. Rats were administered 20 mg DMBA (four weekly gavage doses of 5 mg/rat in sesame oil) and were placed in exposure chambers without MF (see below) for 18 weeks. In a second experiment (“experiment 2”), the effect of MF exposure on breast cancer development and growth was compared in the two substrains. Per substrain, two groups of 45 rats received DMBA at the dosing protocol described above and were either MF-exposed or sham-exposed for 18 weeks (see below). In both experiments, rats were 50–54 days of age at first DMBA application. The DMBA dosage and administration schedule was

that used previously in most of our and the Battelle experiments (17). Because body weight of SD1 and SD2 rats was about the same at time of DMBA application and exhibited only very minor (approximately 6–7%) variation between rats within both substrains, the fixed dose of DMBA (20 mg) per rat corresponded to ~ 100 mg/kg body weight without any substrain difference. DMBA ($\sim 95\%$ purity) was obtained from Sigma (Taufkirchen, Germany).

MF Exposure. The exposure system and the protocol for MF and sham exposure have been described in detail elsewhere (24, 25). In short, rats were exposed in exposure chambers to a horizontally polarized magnetic 50-Hz field with a flux density of 100 μT (*i.e.*, 1 Gauss) root mean square for 18 weeks. Identical but nonenergized exposure chambers were used for sham-exposure of control rats in the same room. Three exposure chambers with room for four cages each were used for MF exposure and three exposure chambers (at the other side of the room) for sham controls (25). Each cage could be used for up to 9 rats, allowing a maximal sample size of 108 MF-exposed and 108 sham-exposed rats. Sham-exposed rats received a stray MF field from the energized coils, which was calculated (and measured) to be ~ 0.1 μT in the volume of the sham exposure chambers.

For the animal experiments, the rats were randomly divided into groups of 4–9 animals, depending on the sample size of the experiment. At the onset of the MF experiment, at which the rats were 50–54 days of age, they were brought into the room with the exposure chambers, placed in their home cage into the exposure chambers, and MF exposure was started for 24 h/day (minus time for weighing, cage cleaning, and cage rotation) 7 days/week except for the concurrent sham controls, which were placed in identical exposure chambers without MF. Rats of experiments 1 and 2 received the first application of DMBA (5 mg/rat) at the first day of MF or sham exposure. Rats were housed 4–9/cage within the exposure or sham exposure chambers under controlled conditions of temperature (23–24 $^{\circ}\text{C}$), humidity ($\sim 50\%$), and light (12-h dark/light cycle; light off at 6 p.m.); food (Altromin standard rat diet) and water were available *ad libitum*. Light intensity produced by the artificial white light in the room with the exposure system varied between 16 and 35 lux (measured by a luxmeter in the exposure chambers). In the dark period, the room was weakly illuminated by dim red light, which led to a light intensity of below 1 lux (measured in the exposure chambers).

Animals were weighed once per week; cage cleaning was done three times a week; cage rotation in the exposure chambers was done once a week. The 50-Hz MF in the exposure chambers was measured twice per week with a μT -Vector2 meter (Physical Systems, Bradenton, Florida). In addition, the current generating the MF was measured continuously by a Clamp On Leak Hi Tester (Hioki E.E. Corp., Nagano, Japan) and recorded by a computer every 5 s. The mean current value of 1 min, and the minimum and maximum values of the last 24 h were continuously recalculated and were visible at a monitor for direct control of stable exposure conditions during the experiments and were saved on a computer for retrospective analysis. The system and the software for controlling the current generating the MF were created by the Forschungsverbund Elektromagnetische Verträglichkeit Biologischer Systeme (Department of High Voltage Engineering, Technical University, Braunschweig, Germany). During the MF experiments, all of the field measurements were done by a person not involved in the animal experimentation.

For the DMBA experiments (experiments 1 and 2), SD1 and SD2 rats were sham- or MF-exposed together over the same period. For experiment 1, SD1 and SD2 rats were sham- exposed from February to June; for experiment 2, SD1 and SD2 rats were MF- or sham-exposed from September to February, so that the experiments were performed in different seasons of the year, which may affect the sensitivity of the mammary gland to DMBA (26). All of the experimental details were the same for both groups in the two experiments.

Quantification of Mammary Tumors. During MF or sham exposure, rats were palpated once per week for the detection of mammary gland tumors in both experiments. The size of palpable tumors was estimated by a rating scale as described recently (27). Furthermore, the location of each tumor among the six mammary complexes of the rat was recorded.

After 18 weeks of MF or sham exposure, all of the rats were killed for necropsy. Rats that had to be necropsied before the end of the exposure period because of large bleeding tumors were included in the pathological examination. The weight of liver and spleen was recorded in all of the animals before fixation. Preparation of the mammary glands was done as described above for the proliferation assays. All of the grossly observed (*i.e.*, macroscopically visible) mammary tumors were recorded, excised, trimmed, and saved for

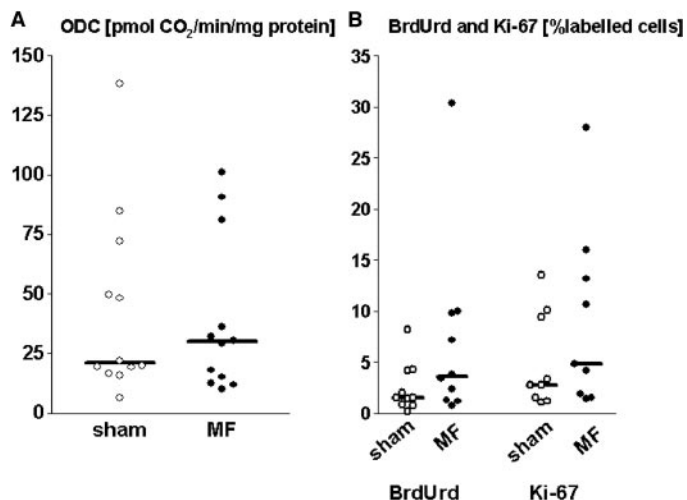


Fig. 1. Effect of MF exposure (50-Hz, 100 μ T, 2 weeks) on ornithine decarboxylase (ODC) activity (A) and bromodeoxyuridine (BrdUrd) or Ki-67 labeling (B) in the cranial thoracic mammary complex (complex 1) of female SD2 rats. Data for ODC were determined in R1 and are shown for 12 MF-exposed and 12 sham-exposed rats. Data for BrdUrd and Ki-67 labeling were determined in L1 of the same rats and are shown for 9–10 MF-exposed and 9–10 sham-exposed rats. Horizontal bars indicate the median. Data from MF- and sham-exposed groups did not significantly differ.

additional histopathological analysis. The size of macroscopically visible mammary tumors was measured by a caliper after dissection, and tumor volume was calculated from the length, width, and depth of tumors on the basis of an ellipse. The mammary tumors were then fixed in 4% phosphate-buffered formalin (pH 7.3). The fixative was changed after 24 h. Small tumors were fixed in total or cut in two halves. For large tumors, 1–2 sections were cut vertical to the surface and to the midline. These tissue samples were embedded in Paraplast, sectioned at 3–4 μ m, and stained routinely with H&E. Neoplastic lesions of the mammary glands were classified by microscopic examination according to Boorman *et al.* (28). The histopathological evaluation was done “blind,” *i.e.*, the examiner was not aware of the group origin of sections.

With respect to the tumors palpated before necropsy, only the neoplasms that were subsequently histologically verified as mammary tumors were used for group comparisons. In experiment 2, only tumors located in the cranial and middle thoracic mammary complexes (L/R1 and L/R2) were used for final evaluation, because previous data from our group have demonstrated that these glands respond much more markedly to MF exposure than more caudal mammary complexes (12), which can be explained by the strong effect of MF exposure on proliferation of the mammary epithelium in these complexes (10, 20).

Statistics. Differences between groups in tumor incidence were determined using Fisher’s exact test and in the mean number, size, and latency to onset of tumors by the Mann-Whitney *U* test. The latter test was also used for calculation of statistical differences in ODC activity or labeling of BrdUrd and Ki-67. Differences in the cumulative proportions of animals with tumors (incidence curves) were calculated by the log-rank test in which animals that died or were sacrificed without tumors were included as censored. Differences between groups in body weight and organ weights were calculated by Student’s *t* test. For correlation analysis, the Spearman method was used. Except where otherwise indicated, all of the statistical tests were used as two-sided tests and a $P < 0.05$ was considered significant.

RESULTS

Effects of MF Exposure on ODC Activity and Cell Proliferation in the SD2 Substrain. As shown in Fig. 1A, MF exposure did not significantly alter the activity of ODC in mammary tissue of the SD2 substrain. Consistent with this finding, MF exposure did not significantly increase the number of BrdUrd- and Ki-67-positive epithelial cells in the mammary tissue of the same rats (Fig. 1B), indicating no change in the proliferative activity of the mammary epithelium compared with sham controls. In controls, ~2% of the epithelial cells of L1 were labeled by BrdUrd, which is comparable with the percentage of BrdUrd-labeled cells determined recently for this mammary complex in SD1 rats (10).

Different Susceptibility of SD Substrains to DMBA (Experiment 1). As shown in Fig. 2A, based on palpation of mammary tumors during the 18 weeks after the first DMBA application, SD2 rats were significantly more sensitive to the carcinogenic effect of DMBA than SD1 rats. Almost all of the SD2 rats (95%) developed palpable mammary tumors, whereas cumulative incidence of such tumors was only 65% in SD1 rats. However, at necropsy, several small mammary tumors were recorded in SD1 rats that had not been recorded during palpation (Fig. 2A), indicating that the main difference between the two substrains was not tumor incidence but tumor growth. Mean tumor volume per rat with tumors was almost twice as high in SD2 compared with SD1 rats (6381 ± 1796 mm³ versus 3681 ± 1519 mm³; mean \pm SE). Tumor multiplicity, *i.e.*, mean number of tumors per tumor-bearing rat, was about the same in both groups (4.1 ± 0.69 in SD1 versus 4.53 ± 1.2 in SD2, respectively). The predominant type of tumors in both groups was invasive adenocarcinomas.

During the 18 weeks of the experiment, palpable mammary tumors were not equally distributed among the six pairs of mammary glands,

Fig. 2. The cumulative proportion of rats from two SD substrains (SD1 and SD2) with mammary tumors as a function of time after 7,12-dimethylbenz(a)anthracene (DMBA) application (incidence curves). Rats were administered 20 mg DMBA (four weekly gavage doses of 5 mg/rat). Group size was 20 rats/group. The left graph (A) shows cumulative tumor incidence calculated from tumors in all six mammary complexes, the right graph (B) tumor incidence calculated from tumors in the cranial and middle thoracic complexes (L/R1 and L/R2). In addition to the data from palpation (weeks 6–18), the percentage of rats with macroscopically visible (and histologically verified) mammary tumors at necropsy (*i.e.*, after 18 weeks of exposure) is shown. With respect to the tumors palpated before necropsy, only neoplasms that were subsequently histologically verified as mammary tumors are shown. *P*s from statistical evaluation of data from the palpation period by the log-rank survival analysis are shown in each graph. * indicate significance of individual differences between groups ($P = 0.0436$).

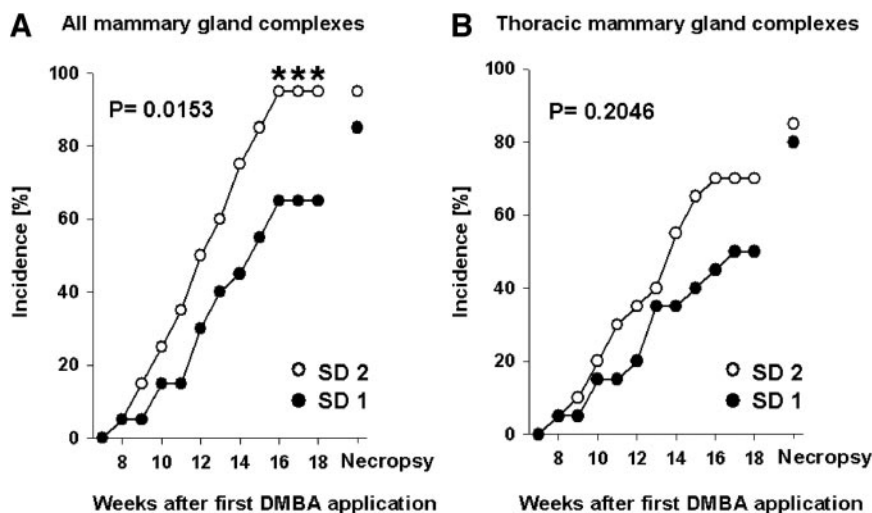
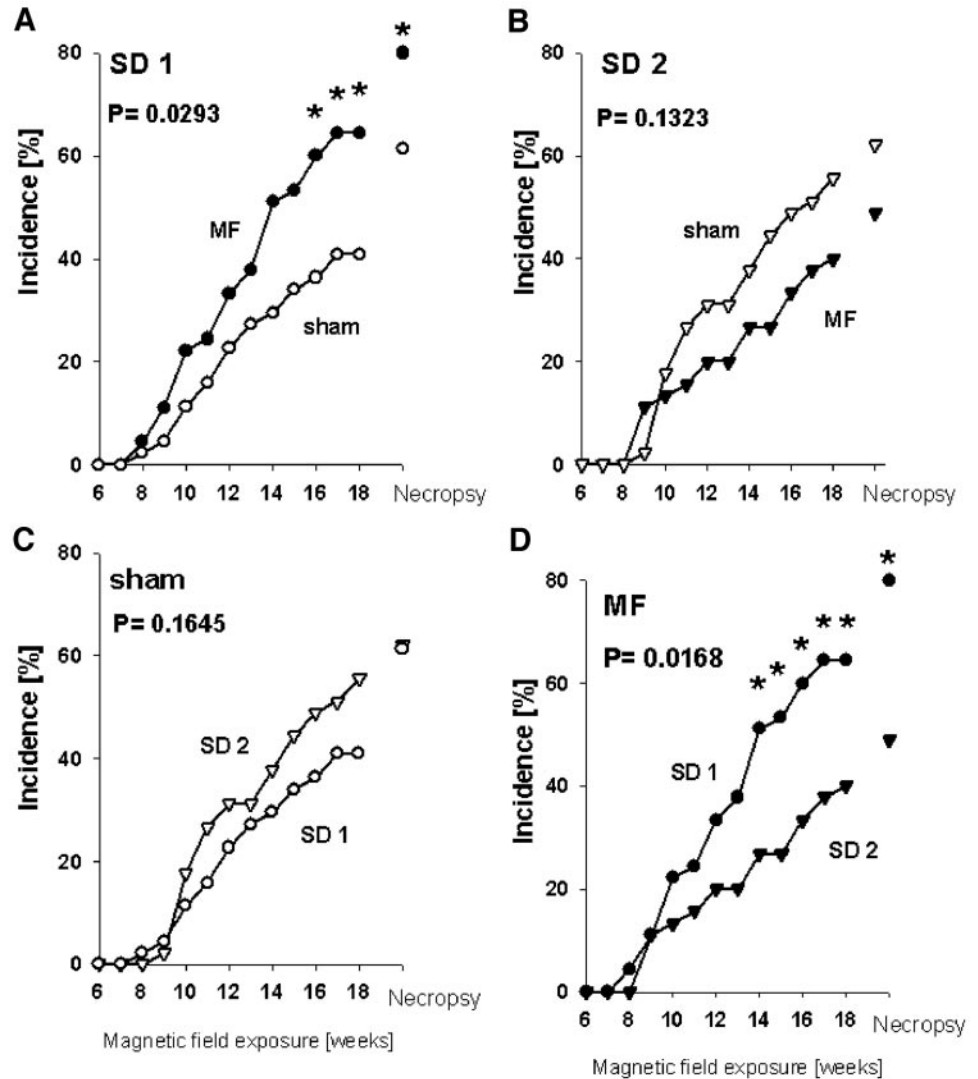


Fig. 3. The cumulative proportion of rats from two SD substrains (SD1 and SD2) with DMBA-induced mammary tumors as a function of duration of MF- or sham-exposure (incidence curves). Rats were administered 20 mg DMBA (four weekly gavage doses of 5 mg/rat); the first application of 5 mg was done at onset of MF- or sham-exposure. Group size was 45 rats/group. In addition to the data from palpation (weeks 6–18), the percentage of rats with macroscopically visible (and histologically verified) mammary tumors at necropsy (*i.e.*, after 18 weeks of exposure) is shown. With respect to the tumors palpated before necropsy, only neoplasms that were subsequently histologically verified as mammary tumors are shown. All tumor data are from the cranial and middle thoracic mammary complexes (L/R1 and L/R2). A shows the data from MF- and sham-exposure for the SD1 substrain; B, data for the SD2 substrain; C, compares data from sham-exposure of SD1 and SD2; and D, data from MF-exposure of SD1 and SD2. *P*s from statistical evaluation of data from the palpation period by the log-rank survival analysis are shown in each graph. * indicate significance of individual differences between groups ($P < 0.05$). The data from necropsy of SD1 rats shown in A were compared by a one-sided test ($P = 0.0324$), because our hypothesis from various previous experiments with SD1 rats was that MF exposure would increase tumor incidence at time of necropsy.



but most tumors occurred in the thoracic glands (Fig. 2B). At time of necropsy, 54% of mammary tumors (38 of 70) were located in L/R1 and L/R2 in SD1 rats compared with 61% (52 of 85 tumors) in SD2 rats. The cumulative incidence of palpable tumors in L/R1 and L/R2 tended to be higher in SD2 compared with SD1 rats (Fig. 2B), although the difference became not statistically significant because of the small sample size.

SD1 and SD2 rats did not differ in body weight at onset of the DMBA experiment (192 ± 2.2 g versus 194 ± 2.4 g). Furthermore, weights of liver and spleen did not differ significantly between groups at time of necropsy (data not illustrated).

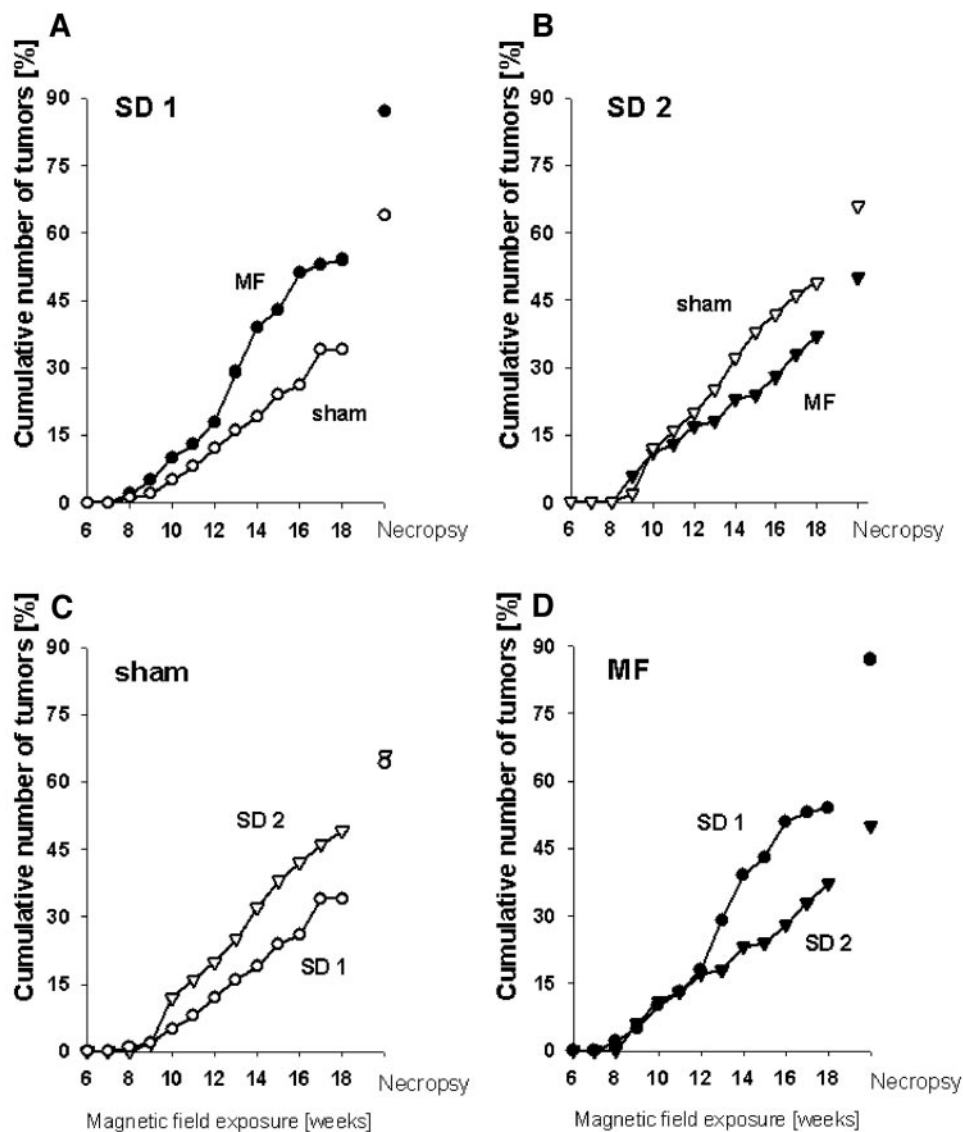
Different Susceptibility of SD Substrains to MF Exposure in the DMBA Model (Experiment 2). Because we have demonstrated previously that the thoracic mammary glands, particularly L/R1 and L/R2, are the most susceptible mammary complexes to MF exposure in the DMBA model (12), analysis of data from these complexes was used for group comparisons between SD1 and SD2 rats in experiment 2. As shown in Fig. 3A, MF exposure significantly increased the percentage of animals with tumors in the SD1 substrain, thus confirming previous experiments in these rats (12, 24, 25, 29). After the first palpation of mammary tumors at 8 weeks, tumor incidence was always above that of sham-exposed rats. Statistical evaluation of the cumulative proportions of SD1 animals with tumors in MF- and sham-exposed groups over the whole period of MF exposure by the

log-rank test yielded a P of 0.0293 (Fig. 3A), indicating that the two groups differed significantly. In contrast, no such difference was observed for SD2 rats, but the MF-exposed group tended to exhibit a lower cumulative tumor incidence than sham controls, which, however, was not statistically significant (Fig. 3B). When cumulative tumor incidence in sham controls was compared, the incidence of palpated tumors tended to be higher in SD2 rats than in SD1 rats (Fig. 3C), similar to the data of experiment 1 (Fig. 2B). A highly significant difference ($P = 0.0168$) was obtained when cumulative tumor incidence during MF exposure was compared between the SD1 and SD2 groups (Fig. 3D).

Fig. 4 illustrates the cumulative number of mammary tumors in the four groups of rats during the 18 weeks of exposure. As could be expected from the incidence curves (Fig. 3), a higher number of mammary tumors was observed in the MF-exposed SD1 group throughout the period of tumor development and growth (Fig. 4A). At time of necropsy, a total of 64 mammary tumors was recorded in the cranial and middle thoracic complexes in the sham-exposed SD1 group, compared with 87 grossly recorded mammary tumors in the MF-exposed SD1 group. In contrast, total number of mammary tumors in MF-exposed SD2 rats was lower than that in sham-exposed SD2 rats (50 versus 66 at time of necropsy; Fig. 4B).

As in experiment 1, analysis of all of the tumors detected in the six mammary complexes in experiment 2 revealed that the thoracic

Fig. 4. The cumulative number of DMBA-induced mammary tumors in rats from two SD substrains (SD1 and SD2) as a function of duration of MF- or sham-exposure. Rats were administered 20 mg DMBA (four weekly gavage doses of 5 mg/rat); the first application of 5 mg was done at onset of MF- or sham-exposure. Group size was 45 rats/group. In addition to the data from palpation (weeks 6–18), the number of macroscopically visible (and histologically verified) mammary tumors at necropsy (*i.e.*, after 18 weeks of exposure) is shown. With respect to the tumors palpated before necropsy, only neoplasms that were subsequently histologically verified as mammary tumors are shown. All tumor data are from the cranial and middle thoracic mammary complexes (L/R1 and L/R2). A shows the data from MF- and sham-exposure for the SD1 substrain; B, data for SD2; C, compares data from sham-exposure of SD1 and SD2; and D, data from MF-exposure of SD1 and SD2.



glands were particularly sensitive to DMBA (data not illustrated). At time of necropsy, 54% of mammary tumors (64 of 118) were located in L/R1 and L/R2 in sham-exposed SD1 rats compared with 50% (66 of 132) in sham-exposed SD2 rats. In MF-exposed rats, 54% of mammary tumors (87 of 160) were located in L/R1 and L/R2 in the SD1 substrain compared with only 36.7% (50 of 136) in SD2, resulting in a highly significant difference between the SD substrains ($P = 0.0034$). Thus, this analysis substantiated the different MF susceptibility of SD1 and SD2 rats.

Tumor multiplicity was not statistically different between groups. On the basis of the data in L/R1 and L/R2, numbers of tumors per tumor-bearing rat were 2.46 ± 0.4 (SD1, sham), 2.49 ± 0.35 (SD1, MF), 2.36 ± 0.31 (SD2, sham), and 2.27 ± 0.36 (SD2, MF), respectively. Similarly, the average tumor volumes did not differ significantly between groups (data not illustrated). However, during palpation, the examiner had the impression that MF-exposed SD1 rats had a higher frequency of large tumors, which may have been concealed during statistical comparison of means because of the high variation in tumor size at time of necropsy. Therefore, we categorized tumors with respect to size in tumors with volumes $>100 \text{ mm}^3$, $>150 \text{ mm}^3$, and $>200 \text{ mm}^3$, respectively. As shown in Fig. 5, which also includes data from experiment 1, a significantly higher proportion of tumors from

MF-exposed SD1 rats had volumes $>200 \text{ mm}^3$ than tumors from respective sham controls at time of necropsy, indicating that MF exposure had enhanced tumor growth in the SD1 substrain.

Tumor latency, *i.e.*, the time to palpation of the first mammary tumor in L/R1 or L/R2, was not significantly different between groups. Average latencies were 12.1 ± 0.84 weeks (SD1, sham), 11.9 ± 0.56 weeks (SD1, MF), 11.5 ± 0.61 weeks (SD2, sham), and 12.9 ± 0.89 weeks (SD2, MF), respectively.

As in experiment 1, the predominant type of histologically verified DMBA-induced mammary tumors at time of necropsy in experiment 2 was invasive adenocarcinomas (Table 1). A part of these tumors could be additionally classified into subtypes as shown in Table 1, but most adenocarcinomas showed a mixed pattern of different subtypes. The frequency of adenocarcinomas arising in fibroadenomas tended to be higher after MF exposure in both SD substrains, the difference between sham- and MF-exposed being statistically significant for SD2 rats (Table 1).

No differences between groups were seen in body weight gain or general behavior during the period of exposure. Average body weight (\pm SE) in MF- and sham-exposed groups at onset of exposure was as follows: SD1 (sham), 186 ± 1.8 g; SD1 (MF), 182 ± 1.7 g; SD2 (sham), 184 ± 1.9 g; and SD2 (MF), 184 ± 2.1 g, respectively. After

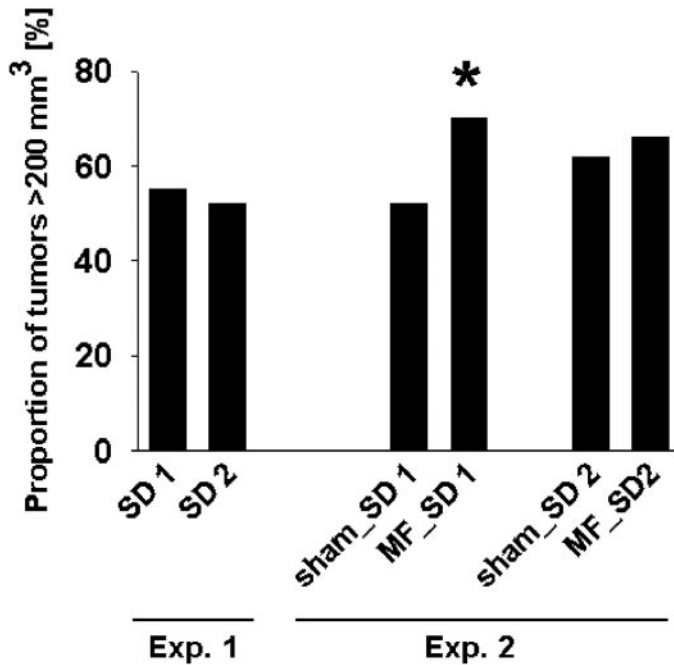


Fig. 5. The proportion of large mammary tumors (volume $>200 \text{ mm}^3$) recorded in the two experiments with induction of mammary tumors by DMBA. In MF-exposed SD1 rats in experiment 2, significantly more tumors had a volume $>200 \text{ mm}^3$ compared with sham controls ($P = 0.0276$; indicated by *). Only tumors recorded in the thoracic mammary complexes (L/R1 and L/R2) were used for the evaluation. Total number of tumors for which tumor volume was recorded was 38 (SD1) and 52 (SD2) in experiment 1, and 64 (SD1, sham), 86 (SD1, MF), 66 (SD2, sham), and 50 (SD2, MF) in experiment 2, respectively.

18 weeks of exposure, body weight was as follows: SD1 (sham), $292 \pm 3.6 \text{ g}$; SD1 (MF), $287 \pm 4.4 \text{ g}$; SD2 (sham), $287 \pm 3.8 \text{ g}$; and SD2 (MF), $292 \pm 5.9 \text{ g}$, respectively. Furthermore, average weights of liver and spleen at time of necropsy did not differ significantly between groups. Liver weights (mean \pm SE) were $11.9 \pm 0.35 \text{ g}$ (SD1, sham), $11.3 \pm 0.27 \text{ g}$ (SD1, MF), $12.0 \pm 0.59 \text{ g}$ (SD2, sham), and $11.7 \pm 0.34 \text{ g}$ (SD2, MF). Spleen weights (mean \pm SE) were $0.75 \pm 0.07 \text{ g}$ (SD1, sham), $0.70 \pm 0.05 \text{ g}$ (SD1, MF), $0.88 \pm 0.08 \text{ g}$ (SD2, sham), and $0.70 \pm 0.05 \text{ g}$ (SD2, MF), respectively. However, in all of the groups, individual rats had abnormally large livers or spleens. A correlation analysis showed a highly significant positive correlation between spleen weight, and both tumor volume ($P < 0.0001$) and number of tumors per rat ($P < 0.002$) for all of the DMBA-treated groups, whereas liver weight and tumor volume/tumor number were only significantly correlated in SD1 rats.

DISCUSSION

To our knowledge, this is the first experimental study demonstrating animal substrain differences in *in vivo* effects of MF exposure on cell proliferation and carcinogenesis under identical conditions at the same laboratory. The difference in the mammary neoplastic response of the SD1 and SD2 substrains to DMBA substantiates previous studies that SD substrains may markedly differ in their sensitivity to this carcinogen (18). Furthermore, as reported previously for ionizing radiation (18), the present data demonstrate that SD substrains may differ in their response to MF exposure. SD2 rats resemble the SD substrain used in the Battelle studies (14–16) in that these rats exhibit a high susceptibility to DMBA but not to MF, whereas the reverse is true for the MF-sensitive SD1 substrain. These substrains can thus serve to evaluate which genetic factors underlie enhanced sensitivity to cocarcinogenic or tumor-promoting effects of MF exposure. Fur-

thermore, our data indicate that replication studies of MF experiments by independent laboratories should consider the impact of genetic diversity and, thus, use the same animal substrain(s) as in the study to be replicated.

Rat strain differences in the susceptibility to DMBA are well established (30–35) and seem to depend on genetic variability in the expression or activity of several mammary carcinoma suppressor and susceptibility genes (34), whereas differences in substrains have been only rarely reported (18, 36). However, it is well known that SD substrains, including SD substrains from the same provider, may show marked differences in a variety of responses (37–42). SD rats are randomly outbred; hence, allelic variations can occur across vendors. Prompted by reports that female SD rats obtained from a United States source and studied in the United States gave a larger and more rapid mammary neoplastic response to ionizing radiation than did female SD rats obtained from a Dutch source and studied in the Netherlands. van Zwieten *et al.* (18) used SD rats from the two sources to study their response to DMBA at the same laboratory under identical conditions. The SD rats from the United States were strikingly more susceptible to DMBA than the Dutch SD substrain, indicating that there are genetic differences between the two SD substrains, which were confirmed by demonstrating dissimilarities in the major histocompatibility haplotypes (18). More recently, a similar difference between United States and European SD substrains in the mammary neoplastic response to DMBA was reported, in that female SD outbred rats obtained from a United States breeder (Charles River, Raleigh, NC) and studied by Battelle in the United States showed a much higher cumulative incidence of mammary tumors than female SD outbred rats obtained from a German subsidiary of Charles River (Sulzfeld) and studied by us in Germany, although the carcinogen dosage and administration schedule were the same in both studies (17). As shown by the present data, the response of outbred SD rats to DMBA may even vary if substrains are obtained from the same breeder within one country.

SD rats from different sources may have little in common with each other besides their names and similarities in pelage, because many of the commercially available animals are outbred and have heterogeneous genetic backgrounds (43, 44). Genetic divergence between outbred subpopulations may arise from a number of processes, including mutation, natural selection, unconscious selection, and random genetic drift (45). By far the most important source of genetic divergence among outbred subpopulations is random genetic drift, especially in small populations. This may also occur within the same large subsidiary of a breeder when subpopulations of outbred rodents are maintained in different areas or breeding colonies over the long term, eventually resulting in genetic drift over time and genetic divergence between colonies.

The present SD2 substrain was initially differentiated by us from SD1 by the lack of MF exposure to increase ODC activity in the thoracic mammary glands. On the basis of these data, our hypothesis

Table 1 Absolute numbers and histopathological diagnosis of grossly recorded mammary gland neoplasias induced by 7,12-dimethylbenz(a)anthracene in sham- and magnetic field-exposed SD1 and SD2 rats in experiment 2

	SD1 Sham exposure	SD1 MF exposure	SD2 Sham exposure	SD2 MF exposure
Mammary tumors (total)	64	87	66	50
Adenocarcinomas	64	86	66	50
Alveolar/solid type	5	2	1	1
Papillary pattern	2	1	1	0
Arising in fibroadenomas	2	7	0	4 ^a
Cribriform pattern	2	2	1	1
Mixed pattern	53	74	63	44

^a Significantly different from sham-exposed SD2 ($P = 0.0322$).

was that SD2 rats are MF-insensitive, which was substantiated by the present experiments demonstrating lack of effects of MF exposure on cell proliferation and in the DMBA model of breast cancer. Whereas SD1 rats, which we have used for MF experiments over >10 years, reproducibly show increased ODC activity (19, 20), enhanced BrdUrd and Ki-67 labeling in the mammary epithelium (10), and enhanced development and growth of DMBA-induced mammary cancer on MF exposure under the experimental conditions chosen for these experiments in our laboratory (12, 24, 25, 29), no such effects were obtained in SD2 rats. As shown recently in SD1 rats (12), the thoracic mammary glands (L/R1, L/R2) are particularly sensitive to MF exposure. It is well known that not all of the mammary glands of virgin female SD rats respond to administration of DMBA in the same fashion; tumor incidence in thoracic mammary glands is higher than in the abdominal glands (33, 46–48). It is thought that this different carcinogenic response is due to the asynchronous development of mammary glands in different topographic areas; thoracic glands lag behind in development and retain a higher concentration of terminal end buds, *i.e.*, the site of origin of mammary carcinomas (35, 48). Recent experiments from our group, using both determination of ODC and different proliferation markers (BrdUrd and Ki-67), indicate that the thoracic glands of SD1 rats are particularly sensitive to increased cell proliferation in response to 50-Hz, 100 μ T MF exposure, which might explain the higher susceptibility of these complexes to cocarcinogenic or tumor-promoting effects of MF exposure (10, 20). Thus, the lack of MF to increase cell proliferation in the mammary glands of SD2 rats would be a likely explanation for the striking difference between SD2 and SD1 rats observed in the present MF effects in the DMBA model.

In the original melatonin hypothesis of Stevens and colleagues (9, 49, 50), MF exposure increases cell proliferation in the mammary epithelium by suppressing the normal nocturnal synthesis of pineal melatonin, which, because of the inhibitory effect of melatonin on estrogen and prolactin production, results in increased levels of these sex hormones, and thereby induces increased proliferation of breast epithelial stem cells at risk for malignant transformation. However, under the same conditions of MF exposure that increased cell proliferation in the mammary gland of SD1 rats, no significant effect on melatonin levels in the pineal gland or mammary tissue were seen in recent experiments (10). In line with this lack of MF exposure to suppress melatonin production, we recently found no increased estrogen or prolactin production in MF-exposed female SD1 rats (51). Thus, differences in the response of pineal melatonin production to MF exposure are not likely to underlie the differences in MF exposure on cell proliferation and DMBA-induced mammary neoplastic response observed in the SD1 and SD2 substrains in the present study.

However, the lack of MF exposure to alter melatonin or sex hormone levels does not exclude that an interaction between MF exposure and melatonin is involved in the effects of MF on mammary cell proliferation. Melatonin exerts a direct antiproliferative effect on estrogen-responsive breast cancer cells and also inhibits the growth of normal mammary tissue (52–55). Melatonin exerts its antiproliferative action by delaying the progression of cells from G₁/G₀ to the S phase of the cell cycle (56). The mechanisms underlying this direct antiproliferative effect of melatonin in the mammary gland involve a melatonin receptor-mediated down-regulation of estrogen receptors, increased expression of the tumor suppressor gene *p53*, a reduction of DNA synthesis, and effects on calcium homeostasis (56–59). Fifty- or 60-Hz MF exposure in the μ T range has been shown by different laboratories to block the antiproliferative effect of melatonin in MCF-7 human breast cancer cells, most likely by uncoupling of signal transduction from melatonin receptors (60–62). Thus, a suppressive action of MF exposure on function rather than concentration of melatonin, *i.e.*, a modified melatonin hypothesis, would be a possible

explanation for findings of increased proliferation in the mammary epithelium in response to MF exposure in SD1 rats (10). Interestingly, similar to the SD substrain differences in MF effects on cell proliferation and mammary carcinogenesis, genetic differences in MCF-7 breast cancer cells from different laboratories were found to influence the effects of MF exposure on the growth-response to melatonin *in vitro* (2, 63).

Apart from melatonin, direct effects of MF exposure on gene expression could be involved in MF effects on the mammary gland (2). MF exposure has been reported to increase the expression of a number of oncogenes, including *c-myc*, in different cell systems (2, 64, 65). The protein products of oncogenes such as *myc* are thought to facilitate progression of the cell through the cell cycle and synthesize DNA in S phase (66), so that MF-induced *myc* expression would be a likely explanation for increased cell proliferation in response to MF exposure as found recently in SD1 rats (10). However, several groups could not reproduce reports on enhanced expression of oncogenes in response to MF exposure (67–70). Similar to the findings from MCF7 cells (2), the genetic background of the cell preparations used in such studies may determine whether or not cells respond to MF exposure. This may finally explain why so many biological experiments with MF exposure yield contradictory results.

In summary, by studying the susceptibility of genetically different substrains of SD rats to DMBA and effects of MF exposure on DMBA-induced mammary carcinogenesis under identical laboratory conditions, we have found a substrain (SD2) that appears to be MF resistant and, thus, clearly differs from the MF-sensitive SD1 substrain used in the present and previous experiments of our group. We now plan to directly compare in our laboratory the SD substrain used by Battelle for MF studies in the United States (2, 14–16) with our MF-sensitive SD1 substrain. Furthermore, we have started to examine other outbred and different inbred rat strains for MF sensitivity. The use of MF-sensitive and -resistant strains or substrains of rats offers a valuable approach to search for genetic factors or genetic predisposition that may underlie the sensitivity to cocarcinogenic or tumor-promoting effects of MF exposure.

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