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Magnetic Field Exposure Increases Cell Proliferation but Does Not Affect Melatonin Levels in the Mammary Gland of Female Sprague Dawley Rats[1]

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ABSTRACT

In line with the possible relationship between electric power and breast cancer risk as well as the underlying "melatonin hypothesis," we have shown previously (Thun-Battersby *et al.*, Cancer Res., *59:* 3627–3633, 1999) that 50-Hz magnetic fields (MFs) of low (100 μTesla) flux density enhance mammary gland tumor development and growth in the 7,12-dimethylbenz(*a*)anthracene model of breast cancer in female Sprague Dawley rats. On the basis of the melatonin hypothesis and previous observations of induction of ornithine decarboxylase in response to MF, we proposed that the effect of MF exposure on mammary carcinogenesis is related to enhanced proliferation of the mammary epithelium. The objective of the present study was to directly assess this proposal by the use of proliferation markers. Female Sprague Dawley rats were MF or sham exposed for 2 weeks at a flux density of 100 μTesla. Proliferation of epithelial cells in the mammary tissue and adjacent skin was examined by *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, melatonin levels were determined after MF or sham exposure in the pineal gland and directly in the mammary tissue. In additional experiments, the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate was used for comparison with the effects of MF exposure. MF exposure significantly enhanced BrdUrd and Ki-67 labeling in the mammary epithelium, indicating a marked increase in cell proliferation. The most pronounced effect on proliferation was seen in the cranial thoracic (or cervical) mammary complexes, in which we previously had seen the most marked effects of MF exposure on mammary carcinogenesis. In contrast to the melatonin hypothesis, melatonin levels in pineal or mammary glands were not affected by MF exposure. Topical application of 12-*O*-tetradecanoylphorbol-13-acetate increased BrdUrd and Ki-67 labeling in epithelial cells of the skin, particularly in hair follicles, but not in the mammary tissue. The data demonstrate that MF exposure results in an increased proliferative activity of the mammary epithelium, which is a likely explanation for the cocarcinogenic or tumor promoting effects of MF exposure observed previously by us in the 7,12-dimethylbenz(*a*)anthracene model of breast cancer.

INTRODUCTION

The hypothesis that the increased breast cancer risk in industrialized countries is related to the increased use of electricity, particularly by the increased exposure to MFs[3] and light-at-night, has attracted a great deal of interest (1–3). Some epidemiological studies found significant associations between MF exposure and breast cancer (4–7), but other studies were negative (8–10), so that it is currently unclear if MF is a significant risk factor for breast cancer (3, 9). We found previously that prolonged exposure to 50-Hz MFs at flux densities in the μT range enhances mammary tumor development and growth in the DMBA model of breast cancer in female SD rats (11–13). In the mammary gland of normal (non-DMBA treated) SD rats, we found that MF exposure increases the activity of ODC, a rate-limiting enzyme in the biosynthesis of polyamines, which are essential for cell proliferation (14, 15). Consistent with the electric power/breast cancer hypothesis of Stevens *et al.* (1, 16, 17), we therefore proposed that MF exposure increases the proliferation of epithelial stem cells in the mammary gland, thereby increasing the sensitivity of these cells to DMBA and promoting the growth of initiated cells. This proposal was indirectly substantiated by the observation that the most pronounced MF effect on tumor development and growth was seen in the cranial thoracic complexes (L/R1) of the rat mammary gland, in which MF also induced the most marked increase in ODC (13, 15).

However, our data have been criticized, because ODC is not a direct marker of cell proliferation and because in our ODC measurements we determined ODC in mammary gland samples including the adjacent skin, so that it remained unclear whether the ODC increase was related to the mammary gland tissue. A variety of cellular studies have reported MF effects on the activity of components of the pathways that regulate cell proliferation, and some *in vitro* studies have found increased proliferation of epithelial cells and lymphocytes in response to MF (18, 19). This prompted us to study whether MF exposure alters mammary epithelial cell proliferation by using proliferation markers, *i.e.*, antibodies to BrdUrd and Ki-67. Whereas BrdUrd, a thymidine analogue incorporated into DNA as 5-bromouracil during the S phase of the cell cycle, can be used to label proliferating cells in the S phase, antibodies against the nuclear proliferation-associated Ki-67 protein can be used to label all of the active parts of the cell division cycle (G_1, S, G_2, and M) and not only those in S phase or mitosis (20–23). Thus, Ki-67 staining provides a means of evaluating the growth fraction (*i.e.*, the number of cells in cycle) of normal and neoplastic cell populations (20). As a positive control for comparison with the effect of MF exposure, we performed experiments with the tumor promoter TPA, which we found previously to induce similar ODC increases after local application onto rat mammary complexes compared with MF exposure (15). Because inhibitory effects of MF exposure on the pineal production of the hormone melatonin has been proposed as a plausible explanation for MF effects on cell proliferation in the mammary gland (1, 2, 17), melatonin was measured in the pineal gland and mammary tissue of MF-exposed rats.

MATERIALS AND METHODS

Animals. Female SD outbred rats were obtained from Charles River (Sulzfeld, Germany) at an age of 44–47 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 strain and age of the rats, and the exposure conditions of the MF experiment were the same as used previously by us in studies on cocarcinogenic effects of MF exposure in the DMBA breast cancer model (13). The experimental protocols used in this study were in line with

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[3] The abbreviations used are: MF, magnetic field; BrdUrd, bromodeoxyuridine; DMBA, 7,12-dimethylbenz(*a*)anthracene; ODC, ornithine decarboxylase; SD, Sprague Dawley; T, Tesla; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HPLC, high-pressure liquid chromatography.

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 the animals used.

MF Exposure. The exposure system and the protocol for MF exposure used for the present experiments 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 2 weeks. In previous experiments, 2 weeks of MF exposure at 100 μT was found to significantly increase ODC in the rat mammary gland (15), which was the reason to use these MF exposure characteristics for the present study. Identical but nonenergized exposure chambers were used for sham exposure of control rats in the same room. 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 nine to twelve animals. At the onset of the MF experiment, at which the rats were ~52 days of age (except otherwise indicated), the groups were brought into the room with the exposure chambers, placed in their home cage in the exposure chambers, and MF exposure was started for 24 h/day (minus time for weighing, cage cleaning, cage rotation) 7 days/week except for the concurrent sham controls, which were placed in identical exposure chambers without MF. Rats were housed four to six per cage within the exposure or sham exposure chambers under controlled conditions of temperature (23–24°C), humidity (~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 <1 lux (measured in the exposure chambers).

Animals were weighed once/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 continuously measured 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 recalculated continuously, 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.

After 2 weeks of MF or sham exposure, all of the rats received an i.v. injection of BrdUrd (50 mg/kg) in the morning. In four rats from the MF group, the i.v. injection failed, therefore these rats were excluded from BrdUrd labeling but included for Ki-67 labeling. After administration of BrdUrd (1 h), 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 (13, 15), BrdUrd and Ki-67 labeling was performed in the cranial thoracic (or cervical; L/R1) and cranial inguinal (L/R5) 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 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.* (26) and Luettig *et al.* (27), and adapted to the mammary gland (see below). In preliminary experiments, i.v. application of BrdUrd proved to result in higher and more consistent BrdUrd incorporation into mammary cells than i.p. administration.

In additional experiments, groups of rats were exposed to MF as described above to determine whether alterations in cell proliferation are related to changes in melatonin levels in pineal gland or mammary tissue. The age at onset of MF (or sham) exposure was ~52 days, except one experiment in which rats were younger (42 days) to examine whether this alters the sensitivity to MF exposure. Because melatonin is predominantly produced by the pineal gland during the night, *i.e.,* in the dark, rats were killed after 2 weeks of sham or MF exposure either during the light phase (at 9:30 a.m.-10:30 a.m.) or during the dark phase (at 12:30 a.m.-1:30 a.m.), avoiding any light exposure other than the dim red light described above. The pineal gland was dissected and immediately deep frozen in liquid nitrogen until analysis (see below). Mammary complexes were dissected as described above; the mammary gland tissue within the s.c. adipose tissue was separated from the adjacent skin and deep frozen until analysis.

Application of TPA. In addition to the MF experiments, we performed "positive control" experiments with the tumor promoter TPA (Sigma Chemical Co., Munich, Germany). Groups of six female rats, age ~65 days at application of TPA (*i.e.,* age-matched with the MF-exposed rats after 2 weeks of MF exposure), were used for these experiments. Three days before TPA application, the region of the mammary glands was depilated, using a depilatory. On the day of TPA application, 30 μl of acetone (either with or without 0.5 μg of TPA) were applied onto the skin covering a mammary complex (L/R5), and rats were killed 4.5 h later for determination of proliferation markers in mammary tissue and adjacent skin. BrdUrd was administered i.v. 1 h before killing the rats. The dose of TPA applied to the skin was chosen from previous dose-response curves in rats (15). Tissue sample preparation was performed as described above for MF exposure.

BrdUrd Immunohistochemistry. Cryostat sections were prepared from the rat mammary tissue samples by cutting through the nipple perpendicular to the plane of the skin surface and were fixed for 10 min in equal parts of methanol and acetone (−20°C). After washing in PBS, the sections were additionally fixed for 30 min in 70% ethanol and air dried. To detect incorporated BrdUrd, DNA was denatured with formamide (Sigma Chemical Co.) and NaOH. Formamide (190 ml) was warmed to 70°C, and 1N NaOH (10 ml) was added. The solutions were then mixed for 8 min. The slides were immersed in this solution for 30 s. After washing with TBS/Tween, the slides were immersed in formamide containing 7.5 mM of trisodium citrate for 15 min. These steps were performed at 70°C. After this, the slides were washed in ice-cold TBS/Tween and fixed in 1% formaldehyde (30 min) and 0.2% glutaraldehyde (10 min). Slides were incubated overnight with the mouse antibody anti-BrdUrd (Becton Dickinson, Mountain View, CA) dissolved in TBS/Tween. After washing with TBS/Tween, the bound antibody was revealed using rabbit antimouse (Dako; Hamburg, Germany) and the mouse antibody complex (alkaline phosphatase antialkaline phosphatase, Dako) for 30 min. Each of the last two steps was repeated for 15 min. Then the organ sections were incubated with a mixture of alkaline phosphatase antialkaline phosphatase substrate (Dako) and fast red for 25 min resulting in red staining of the BrdUrd-positive cells. The slides were counterstained with hematoxylin and mounted in glycergel (Dako).

Ki-67 Immunohistochemistry. Cryostat sections were prepared from the rat mammary tissue samples as described above and were fixed for 10 min in equal parts of methanol and acetone (−20°C). After washing in PBS, the sections were additionally fixed for 20 min in 4% paraformaldehyde [prepared in PBS the day before (pH 7.2) at room temperature], and the slides were also fixed for 30 min in 70% ethanol and air dried. To detect the Ki-67 proliferation antigen, the slides were placed in prewarmed 1 mmol of EDTA buffer (90°C) and incubated for 45 min. After washing first at room temperature and then on ice (10 min), the sections were incubated overnight with a mouse anti Ki-67 antibody (MIB-5;; Dianova, Hamburg, Germany). After washing in TBS, the slides were incubated with the second antibody (rabbit antimouse) and the third mouse antibody complex for 30 min. Then the last two steps were repeated for 15 min, and the Ki-67-positive (proliferating) cells were visualized using fast red. The slides were counterstained with Mayers hemalaun (Merck) and mounted in glycergel (Dako).

Evaluation of BrdUrd and Ki-67 Labeling. The percentage of BrdUrd- and Ki-67-positive cells, *i.e.,* the labeling index, in mammary gland tissue, epidermis (basal cell layer), and hair follicles (hair "bulbs") were 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

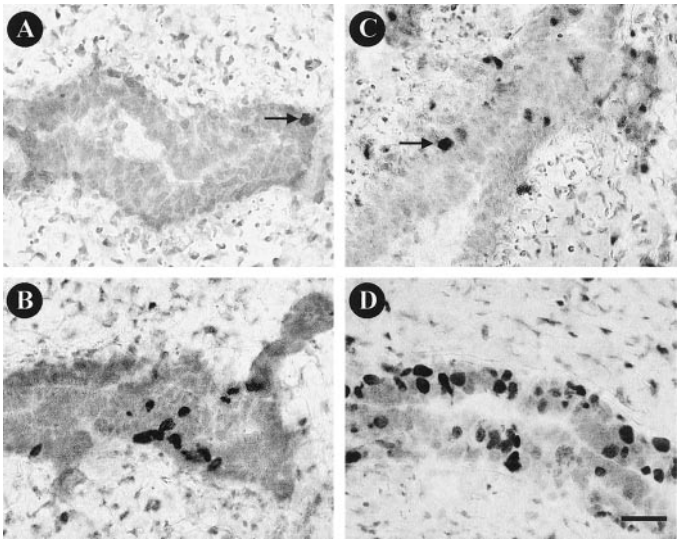

Fig. 1. Cryostat sections of rat mammary gland (cranial thoracic complex). Dark (*black*) cells are positive for BrdUrd (*A* and *B*) or Ki-67 (*C* and *D*) and stained red in original sections. BrdUrd- or Ki-67-positive cells are located in epithelial cells of the mammary gland tissue and not found in the surrounding adipose tissue. *A,* BrdUrd staining of the mammary gland of a sham-exposed rat. The *arrow* points at a BrdUrd-positive cell. *B,* BrdUrd staining of the mammary gland of a MF-exposed rat. Compared with *A,* more cells are BrdUrd-positive. *C,* Ki-67-staining (by MIB-5) of the mammary gland of a sham-exposed rat. The *arrow* points at a Ki-67-positive cell. *D,* Ki-67-staining (by MIB-5) of the mammary gland of a MF-exposed rat. Compared with *C,* more cells are Ki-67 positive. The *calibration bar* indicates 30 μm.

600–900 cells was counted for each section stained for detection of either BrdUrd- or Ki67-positive cells.

Determination of Melatonin in Pineal Gland and Mammary Tissue. For determination of melatonin in rat mammary gland complexes, the method described by Maestroni and Conti (28) was modified. The mammary gland within the s.c. adipose tissue was homogenized for 30 s (Ultra-Turrax; Potter-Elvehjem) and another 10 s (Bandelin Sonoplus HD60; Bandelin Electronic, Berlin, Germany) in an ice-cold mixture (v/v) of 20% methanol and 80% perchloric acid (0.1 N) with a tissue weight:mixture volume 1:20. Thereafter, the homogenate was centrifuged at 3,200 $\times$ *g* for 10 min at 4°C and at 16,000 $\times$ *g* for another 20 min at 4°C. The supernatant was added to a Chromabond C18 column (3 ml; 200 mg; Macherey-Nagel, Düren, Germany) equilibrated with 80% (v/v) methanol followed by two washes with distilled water. After sample addition, the column was additionally washed with 10% (v/v) methanol in water, and melatonin was eluted two times with 200 μl of absolute methanol each. This procedure had an extraction efficiency of 80% when 100 pg of melatonin were added to a pooled supernatant of tissue samples.

For measurement of pineal melatonin, the pineal gland was crushed by a pestle in 75 μl of an ice-cold mixture (v/v) of 20% acetonitrile and 80% perchloric acid (0.1 N). After centrifugation at 14,000 rpm for 2 min, the supernatant was used for melatonin analysis. As commonly done when determining pineal melatonin, pineal glands were not weighed to minimize the time of preparation and the decrease in melatonin, which otherwise occurs when the small glands (about 1–2 mg/gland) are thawed.

Eluted samples were stored at −20°C until analysis. Samples were injected into a HPLC apparatus equipped with a 250-mm stainless steel column (Nucleosil 120–5C18; Macherey-Nagel) and a Rheodyne injector valve with a loop of 50 μl. Electrochemical detection of melatonin was performed using an ESA Coulochem II detector (model 5200A; ESA Inc., Chelmsford, MA) connected to a high sensitivity analytical cell (model 5011; ESA Inc.). The first cell was set at a potential of 300 mV (range: 5 μA) and the second at 600 mV (range: 50 nA). The potential of the guard cell (model 5020; ESA Inc.) was 800 mV. The composition of the mobile phase was 0.05 mM of EDTA, 50 mM of citric acid monohydrate, 50 mM of sodium-acetate, 15% acetonitrile, and 5% methanol; pH was adjusted to 4.3. Flow rate was 1 ml/min. The limit of detection of the used HPLC method was ~10 pg melatonin. Variability of the HPLC analysis was determined by analyzing the same sample seven times. This procedure gave a coefficient of variation of 4.76%. Interassay variability (variability of the extraction method) was 7.69%. The concentration of melatonin in the mammary gland complex was expressed as pg/g of fresh tissue. Pineal melatonin was expressed as pg/gland. Melatonin levels in pineal gland and mammary tissue were determined by a person who was blind to the conditions of the sample (*i.e.*, whether the sample was from a MF-exposed or sham-exposed rat).

Statistical Analyses. For statistical evaluation, each group of MF-exposed rats was compared with its individual sham control group using the Mann-Whitney *U* test. Data from the TPA experiment were also evaluated with the *U* test. In case of more than two groups (*i.e.*, for some of the melatonin data), the Kruskal-Wallis test for nonparametric ANOVA was used with the *U* test as a posthoc test. For comparisons within the same group of rats, the Wilcoxon signed rank test for paired replicates was used. All of the tests were used two-sided, and a $P < 0.05$ was considered significant.

RESULTS

Effects of MF Exposure on Cell Proliferation. Under control (sham-exposed) conditions, *in vivo* BrdUrd labeling showed the characteristic profile for mammary tissue and adjacent skin (Fig. 1). In the cranial thoracic mammary complex (L/R1), ~2% of the epithelial cells of the mammary tissue were stained (Fig. 2), whereas only ~1% of mammary epithelial cells were stained in the cranial inguinal (L/R5) complexes (Fig. 3). The percentage of BrdUrd-labeled epidermal cells in the hair follicles (~3%) and skin (~3%) above the glandular tissue was independent of the topographical location of the mammary complex (Figs. 2 and 3). Ki-67 labeling with MIB-5 resulted in an intensive nuclear staining of epithelial cells, and a much higher labeling index in mammary tissue and skin than BrdUrd labeling (Figs. 1–3). In the cranial thoracic complex, 5% of the epithelial cells of the mammary tissue and ~13% of epidermal cells were labeled by MIB-5 (Fig. 2). Again, a lower labeling index was

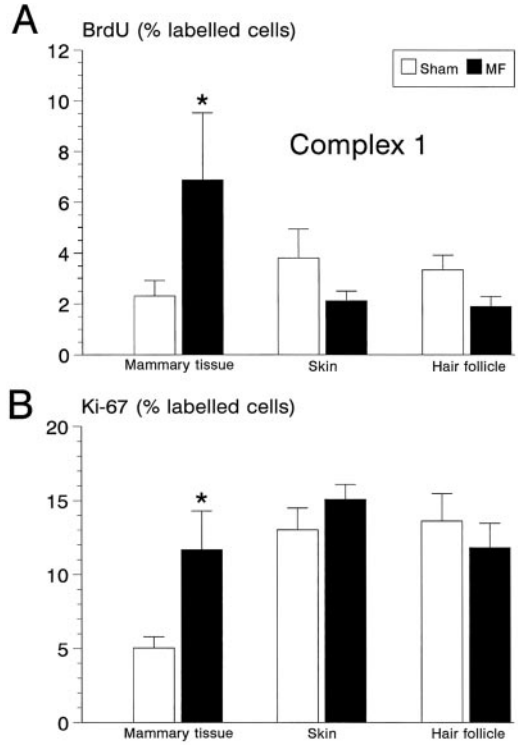

Fig. 2. Effect of MF exposure (50-Hz, 100 μT) on BrdUrd and Ki-67 labeling in the cranial thoracic mammary complex (*Complex 1*) of female SD rats. Groups of nine rats were either sham exposed or MF exposed for 2 weeks. Data are means; *bars,* ±SE; significant increases of BrdUrd- or Ki-67-labeled epithelial cells are indicated by * ($P = 0.0415$ for BrdUrd and 0.0122 for Ki-67, respectively).

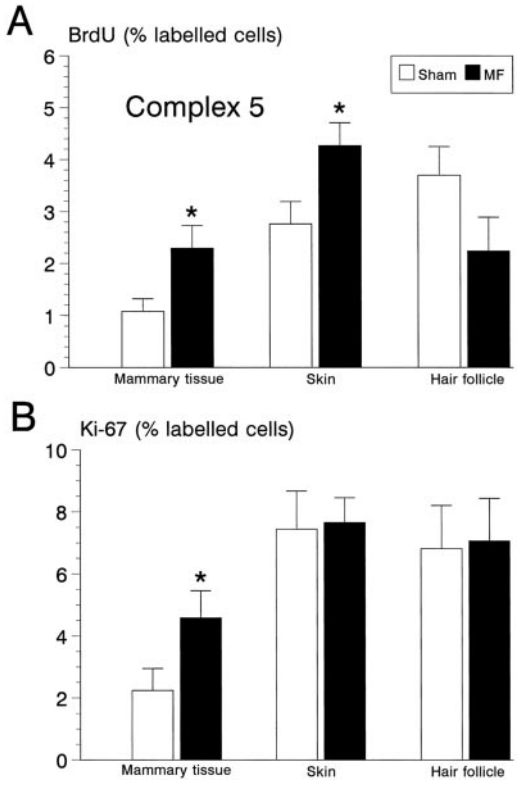

Fig. 3. Effect of MF exposure (50-Hz, 100 μT) on BrdUrd and Ki-67 labeling in the cranial inguinal mammary complex (*Complex 5*) of female SD rats. Groups of nine rats were either sham exposed or MF exposed for 2 weeks. Data are means; *bars*, ±SE; significant increases of BrdUrd- or Ki-67-labeled epithelial cells are indicated by ∗ ($P = 0.0326$ for BrdUrd in mammary tissue and 0.0415 in skin and $P = 0.0466$ for Ki-67 in mammary tissue, respectively).

found in the cranial inguinal complexes (Fig. 3), where only ~2% of the mammary epithelial cells were labeled, with the difference to L/R1 being significant ($P = 0.0177$). The Ki-67 labeling index for the epidermal cells in the skin adjacent to the mammary tissue of L/R5 (~7%) was also significantly lower compared with L/R1 ($P = 0.012$).

MF exposure significantly increased the number of BrdUrd- and Ki-67-positive epithelial cells in both mammary complexes (Figs. 1–3). In the cranial thoracic complex, the BrdUrd- and Ki-67-labeling index in the glandular tissue increased by 200% (BrdUrd) and 130% (Ki-67) on average. In the cranial inguinal complex, respective MF-induced average increases were 112% (BrdUrd) and 104% (Ki-67). Labeling of epithelial cells in skin and hair follicles was not significantly altered by MF exposure except for a small (50%) but statistically significant increase of BrdUrd labeling in the skin adjacent to the cranial inguinal complex (Fig. 3).

As shown previously for the conditions of MF exposure used in the present study (13), MF exposure had no effect on body weight or general behavior of the rats.

Effects of MF Exposure on Melatonin Levels in Pineal Gland and Mammary Gland. The same MF exposure conditions shown to significantly increase BrdUrd and Ki-67 labeling in mammary complexes did not significantly alter melatonin levels in the pineal gland or mammary tissue (Fig. 4). In the pineal gland, we determined the well-known circadian rhythm of melatonin production with highest levels in the dark period and lowest levels in the light period in both sham-exposed and MF-exposed groups without difference between groups (Fig. 4*A*). Interestingly, this marked difference in melatonin levels between dark and light was not seen in the mammary gland (Fig. 4*B*). Because in a first experiment, melatonin levels in the mammary gland tended to be higher in MF exposed rats, we repeated the experiment with a larger group of animals and found about the same melatonin levels in both sham-exposed and MF-exposed rats when determined during the light phase (Fig. 4*B*).

To examine whether melatonin levels differ between mammary complexes, we compared the cranial thoracic complex (L/R1) with one of the abdomino-inguinal complexes (L/R4) and found significantly higher melatonin levels in the thoracic complex (Fig. 4*B*). However, it should be noted that rats from the experiment with melatonin determination in L/R1 were younger (42 days) at the onset of MF and sham exposure than the other groups, which were ~52 days of age at onset of MF and sham exposure. MF exposure had no influence on melatonin levels in L/R1, indicating that age at onset of exposure was not an important factor for MF exposure to affect melatonin levels.

Effects of Local Application of TPA on Cell Proliferation. Local application of the tumor promoter TPA onto the skin covering an inguinal mammary complex (L/R5) did not significantly increase BrdUrd or Ki-67 labeling in the skin or adjacent mammary tissue

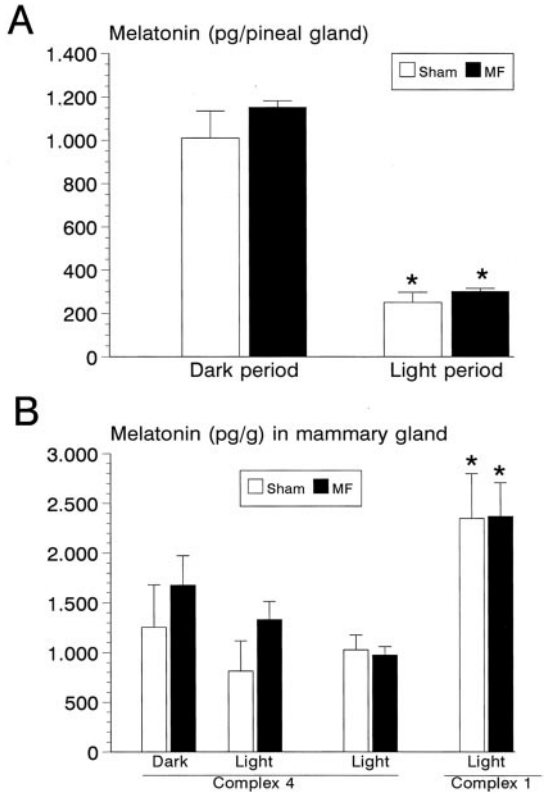

Fig. 4. Effect of MF exposure (50-Hz, 100 μT) on melatonin levels in pineal gland and mammary gland of female SD rats. Groups of rats were either sham exposed or MF exposed for 2 weeks. In a first experiment, the rats were either killed in dark period (at ~1 a.m.) or in the light period (at ~10 a.m.), and melatonin was determined in the pineal gland and in the abdominal mammary complexes (*Complex 4*). Data are means of 4–5 (pineal gland in the dark), 5–6 (pineal gland in the light), and 6 (all mammary data) rats, respectively; *bars*, ±SE. Statistical analysis of pineal data by ANOVA and posthoc testing indicated that melatonin levels in the dark were significantly higher than in the light ($P = 0.002$; indicated by ∗) but that melatonin levels were not affected by MF exposure. Respective analysis of mammary melatonin levels indicated no significant difference between day and night and no effect of MF exposure. The experiment on mammary melatonin levels in the light period was repeated in two other groups of rats ($n = 12$/group), again failing to demonstrate any effect of MF exposure (data are illustrated as the second pair of columns shown for complex 4 and light). Furthermore, in additional groups of rats (8–9/group), mammary melatonin levels were determined in the cranial thoracic complex (*Complex 1*) of MF- or sham-exposed rats, again without any difference between groups. However, statistical comparison of all mammary melatonin groups by ANOVA indicated that melatonin levels in complex 1 were significantly higher than in complex 4 ($P = 0.0007$; indicated by ∗), independent of whether MF or sham exposed. It should be noted that melatonin levels in the pineal gland are expressed per gland (average weight about 1–2 mg), whereas melatonin levels in the mammary tissue are calculated per gram.

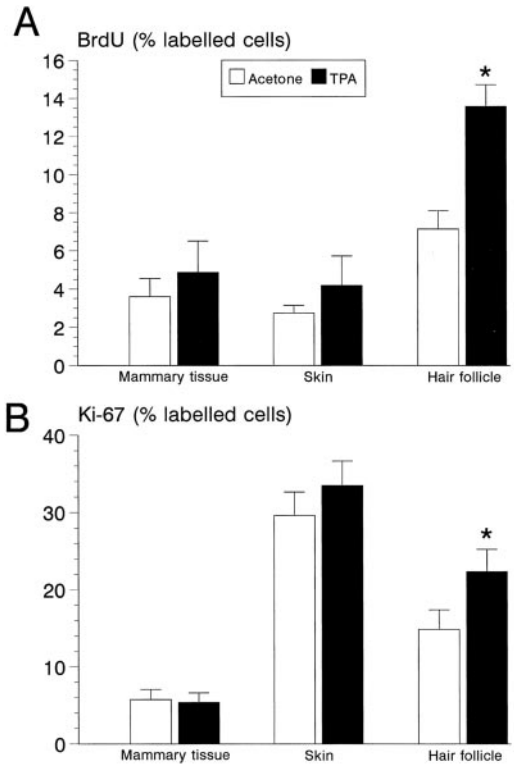

Fig. 5. Effect of local application of acetone with or without TPA (0.5 μg) on BrdUrd and Ki-67 labeling in the cranial inguinal mammary complex (L/R5) of female SD rats. Acetone (vehicle control) or acetone with TPA were locally applied onto the skin covering the mammary complex, and rats were killed after 4.5 h. Data are means of 6 (acetone) or 5 (TPA) rats; *bars*, ±SE. Significant differences between groups are indicated by * ($P = 0.0195$ for BrdUrd and 0.0326 for Ki-67, respectively).

compared with vehicle controls, although there was a tendency for increased labeling of epidermal cells in TPA-treated rats (Fig. 5). TPA application markedly and statistically significantly increased both BrdUrd and Ki-67 labeling in the epidermal cells of the hair follicles (Figs. 5 and 6). It has to be noted that acetone, which was used as a solvent for TPA, seemed to exert effects of its own when applied onto a mammary complex in controls (Fig. 5). Thus, compared with the L/R 5 sham controls of the MF experiments (Fig. 3), most of the values determined for BrdUrd and Ki-67 labeling after application of acetone were significantly higher. The most marked difference was seen for the percentage of Ki-67-labeled cells in the skin, which was 29.6 ± 2.98% in the skin after acetone compared with 7.44 ± 3.49% in naive (not acetone treated) controls ($P < 0.0001$), indicating that acetone itself induced a marked proliferation of epithelial cells, which could have masked part of the effects of TPA.

DISCUSSION

To our knowledge, the present data provide the first direct evidence that MF exposure enhances the proliferation of epithelial cells in the mammary gland. In previous experiments in which the activity of ODC had been determined in homogenates of mammary tissue and adjacent skin, MF exposure (50-Hz, 100 μT) for 2 weeks significantly increased ODC, suggesting an increased cell proliferation (15). By use of *in vivo* BrdUrd labeling and *in situ* Ki-67 labeling of proliferating cells, the present study demonstrates that MF exposure significantly increases the percentage of proliferating cells in the mammary epithelium, whereas no robust effects on epidermal cell proliferation in the skin or hair follicles were seen. Ki-67 labeling is widely used for broad recognition of proliferative activity and as a measure of growth fraction of normal and neoplastic cell populations (20, 23). Because antibodies against Ki-67 such as MIB-5 label all of the active parts of the cell division cycle, the Ki-67 labeling index is usually greater than the BrdUrd labeling index (22), which was also seen in the present study. However, whereas an increased BrdUrd labeling index is considered a robust indicator of an increased fraction of cells in S phase, increased Ki-67 labeling does not necessarily mean that an increased number of cells go through all parts of the cell cycle, including the S phase, so that BrdUrd and Ki-67 labeling is not always correlated (22). This was one reason to use both proliferation markers for the present study. MF exposure significantly increased Ki-67 and BrdUrd labeling in mammary tissue, clearly indicating an increased number of cycling cells in response to MF exposure.

A selective effect of MF exposure on proliferation of epithelial cells in mammary tissue as seen in the present experiments would be in line with the "melatonin hypothesis" of Stevens (1, 16, 17). On the basis of this hypothesis, MF exposure suppresses 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 induce increased proliferation of breast epithelial stem cells at risk for malignant transformation (17). However, under the same conditions of MF exposure that increased cell proliferation in the mammary gland, no significant effect on melatonin levels in the pineal gland were seen in the present experiments. Furthermore, melatonin levels in the mammary tissue were not affected by MF exposure. Thus, consistent with several recent studies on melatonin levels in pineal gland or plasma of rodents (29–31), MF exposure failed to cause any reduction of melatonin levels, which could explain the marked proliferative response of the mammary gland to MF exposure. 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 SD rats (30).

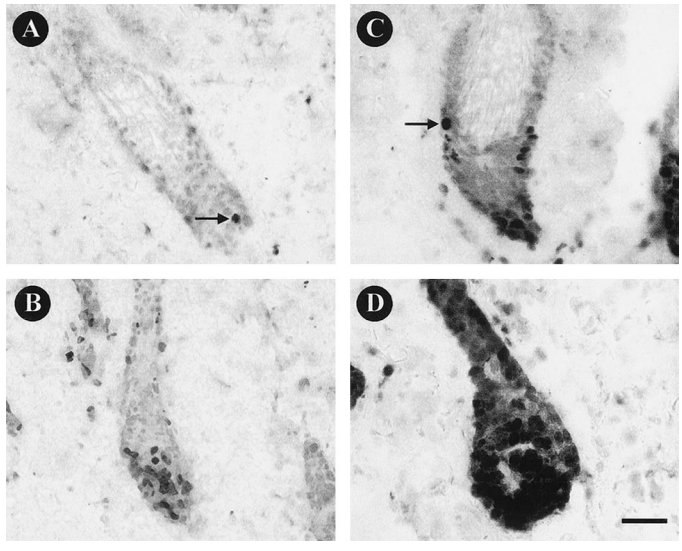

Fig. 6. Cryostat sections of hair follicles (region of the cranial inguinal mammary gland complex). Dark (*black*) cells are positive for BrdUrd (*A* and *B*) or Ki-67 (*C* and *D*) and stained red in original sections. BrdUrd- and Ki-67-positive cells are predominantly located in the hair bulb indicating a higher proliferation in this area. *A*, BrdUrd staining of hair follicles 4.5 h after acetone application (30 μl onto the shaved skin). The *arrow* points at a BrdUrd-positive cell. *B*, BrdUrd-staining of hair follicles 4.5 h after TPA application (0.5 μg TPA in 30 μl acetone onto the shaved skin). In comparison to *A*, more cells are BrdUrd-positive. *C*, Ki-67-staining (by MIB-5) of hair follicles 4.5 h after acetone application (30 μl onto the shaved skin). The *arrow* points at a Ki-67-positive cell. *D*, Ki-67 staining (by MIB-5) of hair follicles 4.5 h after TPA application (0.5 μg TPA in 30 μl acetone onto the shaved skin). In comparison to *C*, more cells are Ki-67-positive. The *calibration bar* indicates 30 μm.

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 (32–35). Melatonin exerts its antiproliferative action by delaying the progression of cells from G_1/G_0 to the S phase of the cell cycle (36). 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 (36–39). MF exposure in the μT range has been shown to block the antiproliferative effect of melatonin in MCF-7 breast cancer cells, most likely by uncoupling of signal transduction from melatonin receptors (40–42). 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 the present findings of increased proliferation in the mammary epithelium in response to MF exposure.

Interestingly, in contrast to the well-known difference in pineal and plasma melatonin levels between night and day, no such difference was seen in melatonin levels in the mammary gland of sham- or MF-exposed rats. This might be explained by accumulation of the lipophilic hormone in the mamma. Indeed, compared with rat plasma, in which melatonin levels typically range from ~50 pg/ml (and lower) during the light period to 100–300 pg/ml during the dark period (30), the levels of melatonin in the mammary gland were much higher, reaching concentrations up to 3000 pg/g, without any significant difference between day and night. To our knowledge, day:night variation in mammary melatonin levels has not been studied before. In a previous study on melatonin in normal human breast tissue, Maestroni and Conti (28) reported average levels of ~7000 pg/g. Similar values were found in neoplastic tissue of the breast (28). In breast cancer tissue, the melatonin concentration correlated positively with the estrogen receptor status but inversely with the nuclear grade of the tumor (28). The present data in normal mammary tissue show that, at least in the rat, the melatonin concentration also depends on the location of the mammary tissue, with highest values seen in the cranial thoracic complexes.

Apart from melatonin, direct effects of MF exposure on gene expression could be involved in the present findings of MF-induced increase in cell proliferation in the mammary gland. MF exposure has been reported to increase the expression of a number of oncogenes, including c-*myc*, in different cell systems (19, 43). 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 (44) so that MF-induced myc expression would be a likely explanation for the present findings. However, some groups could not reproduce reports on enhanced expression of oncogenes in response to MF exposure (45–47). Of particular interest for the recent findings on mammary cell proliferation is a previous study on human breast epithelial cells in which 60-Hz MF exposure at flux densities of 10 μT, 100 μT, or 1 mT did not alter transcript levels of c-*myc* and a battery of other cancer-associated genes (48). We are currently studying whether *in vivo* MF exposure of female SD rats alters the expression and mutation rates of oncogenes in the mammary gland.

The tumor promoter TPA is known to increase the expression of c-*myc* and other oncogenes and to induce epidermal ODC, most likely by activation of protein kinase C (49–52). The enzyme ODC is one of the enzymes necessary for progression of cells into S phase, and, thus, is a critical enzyme in the regulation of DNA replication and cell proliferation (53). We reported recently that *in vivo* application of TPA onto the skin covering a rat mammary complex significantly increases ODC in homogenates of mammary tissue and adjacent skin (15). Because a similar effect on ODC was seen after MF exposure, TPA was used as a "positive control" for the present experiments. However, in contrast to MF exposure, TPA did not increase cell proliferation in the mammary tissue but only in the adjacent skin, particularly in the hair follicles, indicating that TPA did not penetrate into the mammary tissue within the interval (4.5 h) after application used for the TPA experiment. The increased BrdUrd and Ki-67 labeling of epithelial cells of the hair follicles after TPA application onto the skin is consistent with previous observations on growth induction of hair follicles by TPA (54–56). In line with previous experiments on ODC activation (15), acetone, which was used as a vehicle for TPA, exerted skin irritant effects of its own, resulting in a proliferative response of epidermal cells. The skin irritation after local application of acetone is a result of dehydration of the horny layer (57, 58).

In contrast to local application of TPA, MF exposure did not alter epithelial proliferation in hair follicles but predominantly increased cell proliferation in the mammary epithelium. In contrast to limited drug distribution after local application of drugs such as TPA, MF distributes without any restriction throughout the body during whole animal exposure (59). Thus, if MF exposure would induce cell proliferation independent of the specific type of tissue, one would expect an increase of BrdUrd and Ki-67 labeling in epithelial cells of both mammary tissue and adjacent skin (including hair follicles), which, however, was not found in the present experiments, except for a small increase of BrdUrd staining in the skin covering the inguinal mammary complexes. In a previous study in which we determined ODC activity after MF exposure of female SD rats in several tissues, including mamma, liver, spleen, small intestine, bone marrow, and epidermis of the ear, ODC increases were only seen in mammary complexes (80–120% above sham controls) and, to a much lesser extent, in the spleen (42). After intragastric application of DMBA, which was used for comparison, ODC increases of similar magnitude were seen in the mammary complexes, spleen, and intestine. We concluded from these observations that MF exposure induces ODC predominantly in the mamma, which would be in line with the melatonin hypothesis discussed above. However, in a recent study of Kaicer and Mandeville (60), in which female Fischer F344/N rats were exposed to 60-Hz MFs of different flux densities (2, 20, 200, or 2000 μT) for 5 or 15 weeks and ODC was determined in the spleen, kidney, small intestine, liver, spinal cord, and brain, significant increases were found in both the small intestine and spinal cord. The magnitude of the MF-induced increases in these tissues was similar to those induced by the carcinogen ethylnitrosourea, but the latter compound induced ODC in all of the tissues studied. These data indicate that increased cell proliferation after MF exposure, although not occurring throughout all of the tissues, is not restricted to the mammary gland.

Because of our recent observation that the cranial thoracic (or cervical) mammary complexes are particularly sensitive to ODC activation by MF exposure (15), we examined BrdUrd and Ki-67 staining in different mammary complexes of rats after sham or MF exposure. After sham exposure, a higher number of epithelial cells was stained in the cranial thoracic complex compared with an inguinal complex. This is consistent with previous observations on the development of mammary complexes in female rats of the age used in the present experiments in that thoracic glands lag behind in development and retain a higher amount of actively proliferating epithelium than glands located in the abdomino-inguinal area (61, 62). This also explains why the thoracic glands are particularly sensitive to experimental induction of mammary tumors (61, 62). MF exposure significantly increased the number of BrdUrd- and Ki-67-labeled cells in

both the cranial thoracic and the cranial inguinal mammary complexes, but the percentage of increase in BrdUrd labeling in the cranial thoracic complexes was about twice as high as the increase in the inguinal complexes. This enhanced sensitivity of the cranial thoracic complexes to MF exposure is consistent with observations from the DMBA rat model of breast cancer in which we found the most marked MF effect on tumor development and growth in these mammary complexes (13).

In an attempt to replicate our previous MF studies in the DMBA model, a similar experimental protocol was used in a study conducted by Battelle in the United States (63, 64). In contrast to our data, the Battelle studies found no evidence for a cocarcinogenic or tumor-promoting effect of MF exposure. The investigators from the two groups recently discussed differences between their studies that might explain the apparent discrepancies between the results of MF exposure (65). Probably the most important difference is the use of different substrains of SD rats; the United States rats were much more susceptible to DMBA but possibly less sensitive to MF than the rats used in our studies, including the present experiments. It has been demonstrated previously that there are inherent differences between substrains of SD outbred 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 (66). Thus, the use of different SD substrains offers a valuable approach to search for genetic factors or genetic predisposition that may underlie enhanced sensitivity to cocarcinogenic or tumor-promoting effects of MF exposure. We currently compare the SD substrain used for the present experiments with other SD substrains both in terms of MF effects on cell proliferation in the mammary gland, and MF effects on mammary tumor development and growth in the DMBA model. Our aim is find a SD substrain that differs in MF effects from the MF-sensitive substrain used for the present and previous experiments in our laboratory to investigate what accounts for the different MF effects in different SD substrains. In this respect it would also be important to directly compare the SD substrain used by Battelle in the United States with our MF-sensitive SD substrain.

In conclusion, by using two different proliferation markers, the present study demonstrates that, at least under the conditions of our experimental protocol, MF exposure significantly enhances proliferation in the mammary epithelium of female SD rats. This effect of MF exposure occurred in the absence of any alteration in pineal or mammary melatonin levels. On the basis of numerous previous observations in experimentally induced mammary tumors in rats (61, 62), an increased proliferative activity of the mammary epithelium in response to MF exposure is a likely explanation for the cocarcinogenic or tumor-promoting effects of MF exposure observed previously by us in the DMBA model of breast cancer (13).

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Magnetic Field Exposure Increases Cell Proliferation but Does Not Affect Melatonin Levels in the Mammary Gland of Female Sprague Dawley Rats

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