ANTICANCER RESEARCH *21:* 3887-3892 (2001)

C. DOUGLAS WILLIAMS[1], MARKO S. MARKOV[1], W. ELAINE HARDMAN[2] and IVAN L. CAMERON[3]

[1]*EMF Therapeutics, Inc., 1200 Mountain Creek Road, Suite 160, Chattanooga, TN 37405;*
[2]*Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, LA 70808; and*
[3]*University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284-7762, U.S.A.*

Abstract. *Background: A new approach to cancer therapy based on the application of therapeutic electromagnetic fields (TEMF) has been developed by EMF Therapeutics, Inc., Chattanooga, TN, USA. This study was designed to assess the effect of TEMF on tumor vascularization and growth of murine 16/C mammary adenocarcinoma cells in C3H/HeJ mice. Materials and Methods: Implanted tumors were allowed to grow for seven days until the tumor volume reached 100 mm^3 before treatment was started. Mice (20 per control, 10 per EMF exposed group) received treatment (10 minutes per day with 0, 10 mT, 15 mT or 20 mT) with a 120 pulses per second pulsating magnetic field. Tumor growth was assessed throughout the treatment period. The extent of tumor vascularization was evaluated by immunohistochemical staining for CD31. Results: Exposure to TEMF significantly reduced tumor growth, significantly reduced the percentage of area stained for CD31 indicating a reduction in the extent of vascularization and there was a concomitant increase in the extent of tumor necrosis. Conclusion: A novel TEMF treatment safely reduced growth and vascularization of implanted breast cancers in mice. Implication: TEMF may prove a useful adjuvant to increase the therapeutic index of conventional cancer therapy.*

Angiogenesis is defined as the process of formation and development of new blood vessels from existing blood vessels. Angiogenesis occurs as a tightly regulated physiological process during periods of tissue growth, such as embryonic development, any increase in muscle or fat, during the menstrual cycle and pregnancy, as well as in wound healing. However, neovascularization can contribute to a number of pathological processes such as rheumatoid arthritis, diabetic retinopathy, macular degeneration and tumor growth (1-4). It is safe to say that angiogenesis is an important factor in the maintenance and progression of a number of disease states (1,3-6). Angiogenesis may occur either as a natural response to the underlying disease or as a contributory factor to disease progression.

Antiangiogenic therapy is a relatively new approach to the treatment of solid tumors. Since tumor angiogenesis is a fundamental step in tumor growth (7), any method that inhibits the formation and development of a blood vessel network in tumor tissue may lead to the reduction or cessation of tumor growth. No studies were found in the available literature on the use of magnetic fields to reduce angiogenesis. This study was designed to investigate the potential of pulsating electromagnetic fields to inhibit angiogenesis in an animal tumor model, specifically the murine 16/C mammary adenocarcinoma implanted into the C3H/HeJ mouse.

Correspondence to: C. Douglas Williams, EMF Therapeuticts, Inc., 1200 Mountain Creek Road, Suite 160, Chattanooga, TN 37405, U.S.A.

Key Words: Electromagnetic field, angiogenesis, tumor growth.

Materials and Methods

Animals Animal care and handling were performed at the Southern Research Institute (Birmingham, AL, USA). Fifty female C3H/HeJ mice, approximately six-weeks-old, were obtained from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD, USA). The mice were housed in plastic microisolator cages with sterile hardwood bedding and had free access to a standard laboratory diet and filtered tap water. They were weighed on the day of tumor implantation, on days 8, 10, 14 and 17 after tumor implantation and at sacrifice. Air temperature and relative humidity in the animal rooms were controlled at (24±1)°C and (50±10)%, respectively. The lights were operated on automatic 12-hour light/dark cycles. The ambient magnetic field in the exposure chamber was below 50 μT.

Implantation of tumors. The murine 16/C mammary adenocarcinoma cells (National Cancer Institute collection) were first implanted subcutaneously in C3H/HeJ mice and the resulting tumor was maintained by routine passages *in vivo* in mice prior to implantation (8). The tumors were implanted *via* a single subcutaneous injection in the medial left torso (9) of 30 mg of tumor fragments derived from a primitive tumor (10). All implanted tumor fragments were obtained from passage 5 of the adenocarcinoma cell line. The animals were randomized between control (20 animals) and three treatment groups (10 animals per group) after the tumors reached palpable size.

Magnetic field device. A therapeutic electromagnetic field (TEMF) system having a proprietary signal designed by EMF Therapeutics, Inc. (Chattanooga, TN, USA) was used. The system generates a pulsating half sinewave magnetic field with a frequency of 120 pulses per second

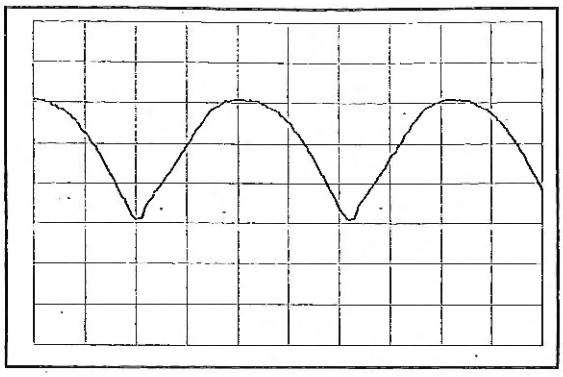

Figure 1. *Representation of the fully rectified 60 Hz signal transferred to 120 pulses per second pulsating magnetic field. The amplitude of the field depends on the current supplying the coil, but the waveform remains the same.*

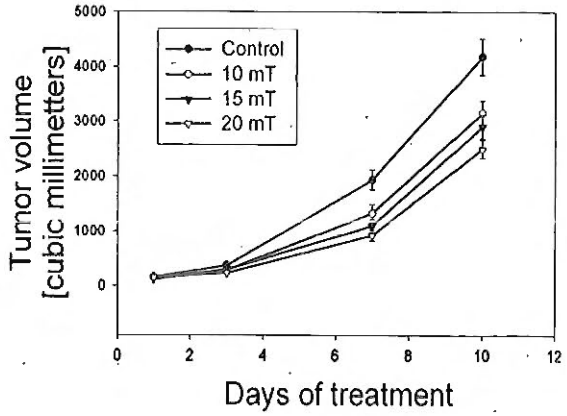

Figure 2. *Volume (Mean ± SEM mm^3) of murine 16/C breast adenocarcinoma in control group and in groups of mice exposed to 10, 15 or 20 mT TEMF for 10 minutes per day for 10 days. Tumors were measured prior to the first exposure (day 8) and on days 10, 14 and 17. ANOVA followed by SNK of the group mean volumes revealed that by day 17 the tumors of the control group were significantly larger than the tumors of all three of the EMF-treated groups. The differences between the tumor volumes of the three EMF-treated groups were not statistically significant.*

(Figure 1). An ellipsoidal coil with 21" large diameter and 14" small diameter is used to deliver the signal to the target. In the experiment reported here, the magnetic flux density measured in the exposure chamber was 10 mT, 15 mT or 20 mT. These values of magnetic field flux density were chosen to investigate the dose-response dependence based upon our previous experience and data (11). A thorough 3-D mapping of the magnetic field was performed for the entire space covered by the coil. The flux density of the magnetic field in the exposure chamber (25 cm long, 10 cm wide and 13 cm high) was consistent within the entire volume of the chamber. The walls of the exposure chamber were perforated to allow air exchange between the exposure chamber and environment. The temperature change inside the exposure chamber at the end of the TEMF treatment did not exceed 1° C, and this change was probably a result of body heat emitted by the mice.

Treatment with magnetic field. The treatment of the animals started after the tumor volume reached approximately 100 mm^3 (evaluated by caliper and calculated by the standard formula discussed below) at day 7 after the tumor was implanted. The animals received magnetic field treatment for 10 minutes daily over 12 consecutive days. The duration of the daily sessions was selected based upon our previous study (11). The control group mice remained in their housing except during the measurement of tumor volume and body weight.

Tumor growth. The tumor volume was calculated by the standard procedure using the formula $V=(AB^2)/2$ where A is the longer diameter, and B is the smaller diameter of the tumor. The same formula for tumor volume was used in a recent publication (12). The length and width of each tumor was measured with calipers on days 8, 10, 14, 17 and 20 after implantation.

Immunohistochemistry. The effect of TEMF on angiogenesis was evaluated by the expression of CD31. CD31 (platelet endothelial cell adhesion molecule, PECAM-1) is a 130 kDa integral membrane protein that mediates cell-to-cell adhesion and is expressed at the surface of endothelial cells. Cross sections of the tumors were cut perpendicular to the underlying muscle layer. The tumor sections for CD31 immunohistochemistry were prepared and stained for CD31 at the Southern Research Institute, (Birmingham, AL, USA). (13). The 12-15 μm-thick, frozen sections were placed on negatively charged ChemMate slides (Ventant, Tucson, AZ, USA), air dried for 24 hours and fixed in acetone. Immunohistochemical staining was performed using a Techmate Automated Staining System and rat anti-mouse CD 31 (Pharmingen, San Diego, USA) monoclonal antibody. Rat IgG2a isotypic serum was used as a negative control. Binding was visualized using biotinylated rabbit anti-rat immunoglobulin followed by streptavidin-horseradish peroxidase and diaminobenzidine.

Table I. *Tumor growth during treatment period - day 8 through day 17. Tumor growth is shown as the increase in tumor volume (volume at day 17 minus volume at day 8).*

Treatment Group	Tumor Growth (mm^3)	SEM (mm^3)
Control	3567	239
10 mT	2945	186
15 mT	2807	169
20 mT	2563	252

Morphometric analyses for the percent of CD31 in viable and in necrotic areas were performed on a subset of tumors randomly sampled from the control group and each treatment group. The cryosectioned tumors previously stained for CD31 reactivity were analyzed using phase contrast microscopy to differentiate necrotic, viable and CD31-stained regions of each tumor. Grid intercept point counting was used to estimate the fraction of an area covered by necrotic, viable or CD31-positive areas.

Statistical analyses. Differences between groups in mean tumor size at each time point and body weights were evaluated by analyses of variance

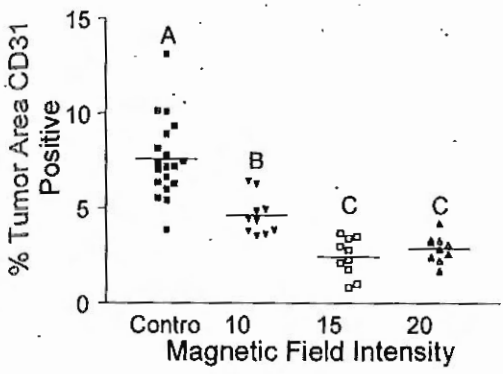

Figure 3. *Effect of exposure for 10 minutes per day for 10 days to a 10, 15 or 20 mT TEMF on the percent of tumor area immunohistochemically - positive for CD31. The mean CD31-positive area of each tumor is shown in the scattergram. Groups with different letters are significantly different. ANOVA followed by SNK statistics revealed the tumors of the control group to have a significantly greater area of CD31-positive vascular tissue than the three EMF-treated groups and the tumors of the 10 mT-exposed group to have a significantly greater area of vascular tissue than the groups exposed to 15 mT or 20 mT.*

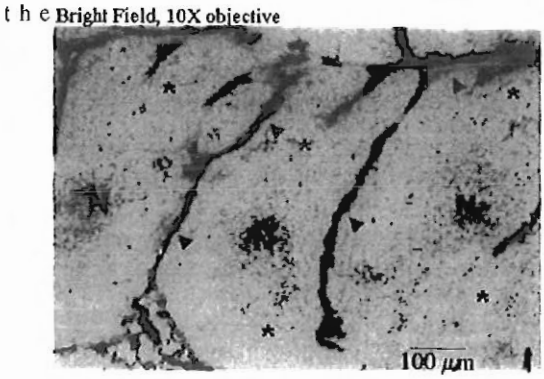

Figure 4. *The photomicrograph is of 12 to 15 μm-thick sections of a 16/C murine mammary adenocarcinoma. The tumor section was subjected to immunohistochemistry to detect the presence of CD31, a marker for blood vessels. Blood vessels (arrowheads) are visualized by the presence of the dark stain for CD 31. Areas of viable tissue are seen adjacent to the blood vessels. Necrotic areas (N) are located further from the blood vessels and contain condensed nuclei and cell fragments.*

(ANOVA) followed by a Student-Newman-Kuels (SNK) multiple range test. Fisher's Exact test was used to compare mortality proportions of the groups. Linear regression analysis was used to compare the relationship of the vascular (CD31-positive) area to the necrotic fraction in the tumors.

Results

Body mass and mortality. There was no significant difference in the increase in body mass of the treatment groups as compared to the control group. Thus, TEMF exposure did not affect the body mass of the mice. All mice in all groups were alive on the 17th day after tumor implantation. By 20 days after implantation, 40% (8 out of 20) of mice in the control group, 40% (4 out of 10) of mice in the 10 mT group, 30% (3 out of 10) of mice in the 15 mT group, but only 10% (1 out of 10) of mice in the 20 mT group had died. Although there was less mortality in the 15 mT and 20 mT groups, the differences were not significantly different by Fisher's Exact test. Thus, as judged by body weight and mortality, the EMF treatment did not result in detrimental effects to the mice.

Tumor growth. Figure 2 is a graph of the mean tumor size of each group for the first 10 days of treatment (days 8 through 17). Days 18 to 20 were not included because of the mortality observed between days 17 and 20. The tumor growth curves begin to diverge as early as two days after the first TEMF treatment. Following 10 days of TEMF treatment, the control group mice had significantly larger tumors than the TEMF-treated mice. The effect on tumor growth is also seen by comparing the gain in tumor volume during the TEMF treatment (volume at day 17 minus volume at day 8 (the day treatment began)). Table I shows that tumor growth during TEMF treatment was significantly less in the treatment groups than in the control group. The greatest reduction from control was found in the 20 mT-treated group ($p < 0.01$). There was no statistically significant difference among the TEMF-treated groups ($p < 0.45$).

Immunohistochemistry. Figure 3 shows the effect of TEMF exposure on the percent of tumor area stained positive for CD 31. All amplitudes of TEMF significantly reduced the percentage of CD31 staining. The percentage of CD31 staining decreased from (7.56±3.35)% in the control group to (4.60±2.20)% in the 10 mT group, (2.42±1.13)% in the 15 mT group and to (2.86±1.06)% in the 20 mT group. ANOVA indicated that CD31 staining was significantly less in all treated groups than in the control group ($p<0.001$). The CD31 staining in the group exposed to 10 mT was significantly less ($p<0.001$) than in the groups exposed to 15 mT or to 20 mT. The difference in CD31 staining between the group exposed to 15 mT and the group exposed to 20 mT was not statistically significant ($p<0.1$).

The change of mean percent of CD31 staining in the TEMF-treated groups *vs.* mean percent in the control group was used to demonstrate the effect of TEMF treatment on angiogenesis in the tumor. CD31 staining in the tumor was significantly decreased 39% by 10 mT TEMF treatment, 68 % by 15 mT TEMF treatment and 62% by 20 mT TEMF compared to staining in the tumors of the control group.

The use of CD31 as a specific marker for blood vessels was confirmed by comparison of bright field with phase contrast microscopy. Phase contrast microscopy of the tumor tissue revealed that viable, necrotic and CD 31-positive areas could be differentiated as seen in Figure 4. Figure 5 illustrates the

significant inverse correlation between vascular area (CD31-positive) and the necrotic area in the tumors. These figures show that the TEMF treatments significantly decreased the vascular density of the tumor and increased the volume density of necrotic tissue in the tumors.

Discussion

Quantifying the CD31 staining was based on the densitometric analysis of the percentages of immunostained areas related to total area of interest (14,15). In normal tissues a strong and homogeneous expression of PECAM-1 can be observed exclusively in endothelial cells of capillaries and in large vessels (16). Therefore, the diminished percentage of CD31 staining demonstrated in Figure 3 should be interpreted as a reduction in vascularity in the tumor.

The results reveal that:

- TEMFs significantly inhibited both angiogenesis and tumor growth;
- The largest inhibition of angiogenesis was observed in the group exposed to 15mT TEMF and the largest inhibition of tumor growth was observed in the group exposed to 20mT TEMF;
- The differences between inhibition of angiogenesis and tumor growth in the 15mT and 20mT groups were not statistically different, thus this study supports the hypothesis that a biological window of efficacy exists within the range of 15-20 mT magnetic field amplitude.

It appears that the inhibition of angiogenesis leads to a reduction in tumor growth. One possible reason for this may be found in the suppressed development of the blood-vessel network which in turn leads to a deficiency in supplying tumor cells with oxygen, ions and nutrients. Cells must be located within about 150 μm of a blood vessel for diffusion to adequately meet the oxygen and nutrient requirements for cell viability (17), thus growth and viability of the tumor strongly depends on angiogenesis. The observed increase in necrotic tissue and decrease in CD31-positive area supports the need for vascularization to maintain tissue viability.

The use of CD31 as a specific marker for blood vessels was confirmed by comparison of bright field with phase contrast microscopy. Observation of the tumor sections by phase contrast microscopy revealed that viable tumor cells were found adjacent to the blood vessels, while areas of tumor at distances greater than 75-150 μm from any blood vessel were necrotic.

The grid intercept method data establishes a relationship between the fraction of CD31-positive area and the necrotic fraction. The results were graphed and analyzed for non-linear regression analysis. A statistically significant ($p<0.001$) negative relationship between the fraction of necrotic tissue and the fraction of vascular tissue was found, that is, the area of necrosis decreased logarithmically as the vascular area increased. This negative relationship was confirmed by the analyses of variance that revealed a significant difference between the necrotic volume in the control samples and samples from animals exposed to TEMF.

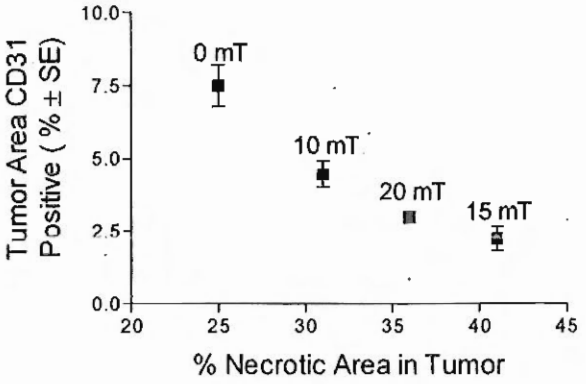

Figure 5. *Relationship between the percent of vascular area (CD31-positive) and the percent of necrotic area in mid sections of the 16/C murine mammary adenocarcinoma. The mean ± SE for each treatment group is shown. Linear regression analyses revealed a significant inverse correlation ($r^2 = 0.94$, p value for significance of slope = 0.03) between vascular area and necrosis.*

The results suggest that therapeutic effects may be achieved by an appropriate selection of the physical parameters of the applied magnetic fields. It has been shown that it is more appropriate to consider biological response to magnetic fields through the hypothesis of "biological windows" instead of dose-response dependence. The biological response probably depends not only on the amplitude of the applied magnetic field but on some other physical characteristics, such as waveform, frequency, repetition rate, presence/absence of the electric field component, *etc.* (18). The results shown in this study confirm reports of an amplitude window in the range of 15-20 mT (19).

At present, we have not hypothesized a mechanism to explain the antiangiogenic effect of the TEMF in this tumor model; however, there are a number of candidate targets for magnetic field action. As tumor cells proliferate into the host tissue, tumor angiogenesis leads to the formation of a new tumor vasculature. Tumor microcirculation originates from the normal host vasculature, but the tumor vessels are more dilated, sacular and tortuous. Furthermore, tumor vasculature has wider intercellular junctions. The extravasion of blood-borne molecules that have reached the tumor vasculature is governed by diffusion and convection (17). TEMF may modify the ability of those molecules to move within the tumor tissue.

The results presented in this paper allow us to conclude that a pulsating magnetic field (120 pps) may inhibit the formation of a blood-vessel network in a growing tumor and that the suppression of the blood-vessel network is probably the main cause of necrotization of the tumor interior and the reduction of tumor growth rate. Whether this effect is repeatable and valid for all types of tumors and whether the

treatment regimen applied to experimental animals will be effective in human tumors remains to be seen. Several studies are ongoing using different protocols to explore these issues.

Although the data from this study reveal that TEMF therapy suppressed vascularization of the tumor and slowed tumor growth, the TEMF-treated tumors did not regress. Is there a rationale for continuation of TEMF therapy research given that the tumors did not regress? Folkman *et al.* (20) make the point that tumors consist of cancer cells whose genome is often too unstable to serve as a fixed therapeutic target and that the cancer cell can acquire drug resistance through natural selection of viable genetic variants within the cancer cell population. The cancer cells in the tumor are known to overexpress factors that stimulate gene expression and the proliferation of endothelial cells thus allowing continued expansion of the tumor. Even though the tumor endothelial cells have up-regulated expression of at least 79 genes compared with "resting" endothelial cells elsewhere in the body, the non-transformed vascular endothelial cells of the tumors have a relatively stable genome. Thus, the tumor endothelial cells are a less variable therapeutic target than are the genomically unstable cancer cells. The cancer cell mass simply can not evade the need for angiogenesis if it is to grow (20). The targeting of tumor endothelium *versus* tumor cancer cells should not be considered as mutually exclusive therapies but anti-angiogenic therapy can, with benefit, be combined with therapies directed towards the cancer cell. Such cancer cell-directed methods include radiation, chemical, immuno- and gene therapies. At present there are multiple reports and ongoing studies on the use of anti-angiogenic chemotherapy for treatment of cancerous tumors (20). The findings from the TEMF study reported herein add TEMF as a simple, safe and non-invasive physical anti-angiogenic tumor therapy that warrants further investigation in combination with currently used cancer cell-directed therapies. Such a combination approach may have additive or synergistic therapeutic value for treating tumors.

Several studies have indicated a synergistic effect between magnetic fields and commonly used chemotherapeutic agents (21 - 24). We are pursuing further studies using different tumor models and exposure conditions to explore the combined action of TEMF and cytostatic agents and to investigate whether the hypothesis of biological windows is applicable to the observed anti-angiogenic and tumor growth effects of TEMF.

References

1 Folkman J and Shing Y: Angiogenesis. J Biol Chem *267:* 10931-10934, 1992.

2 Blood CH and Zetter BR: Tumor interactions with the vasculature: angiogenesis and tumor metastasis. BBA *1032:* 89-118, 1990.

3 Fidler IJ and Ellis LM: The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell *79:* 185-188, 1994.

4 Paku S and Paweletz N: First step of tumor-related angiogenesis. Lab Invest *65:* 334-336, 1991.

5 Folkman J: Clinical application of research on angiogenesis. New England J Med *333:* 1757-1763, 1995.

6 O'Reilly MS, Holmgren L, Chen C *et al:* Angiostatin induces and sustains dormancy of human tumors in mice. Nat Med *2:* 689-692, 1996.

7 Folkman J: Tumor angiogenesis. Adv Cancer Res *43:* 175-203, 1985.

8 Corbett TH, Griswood DP, Jr. Roberts BJ, Peckham JC and Schabel FM: Biology and therapeutic response of a mouse mammary adenocarcinoma (16/C) and its potential as a model for surgical adjuvant chemotherapy. Cancer Treatment Reports *62:* 1471-1488, 1978.

9 Teicher BA (ed): Anticancer Drug Development Guide, 1997, Totowa, New Jersey, Humana Press.

10 Vanzulli S, Cazzaniga S, Braidot MF, Vecchi A, Montovani A, Weinstok de and Calmanovici R: Detection of endothelial cells by MEC 13.3 monoclonal antibody in mice mammary tumors. Biocell *21:* 39-46, 1997.

11 Williams CD and Markov MS: Therapeutic electromagnetic field effects on angiogenesis during tumor growth: a pilot study in mice. Electro-and magnetobiology *20:* 323-329, 2001.

12 De Seze R, Tuffet S, Moreau JM and Veyret B: Effect of 100 mT time varying magnetic fields on the growth of tumors in mice. Bioelectromagnetics *21:* 107-111, 2000.

13 Ruifrok AC: Quantification of immunohistochemical staining by color translation and automated thresholding. Analyt Quant Cytol Histol *19:* 107-113, 1997.

14 De Lisser HM, Christofidou-Solomidou M, Strieter RM, Burdick MD, Robinson CS, Wexler RS, Kerr JS, Garlanda C, Merwin JR, Madri JA and Albelda SM: Involvement of endothelial PECAM-1/CD31 in angiogenesis. Am J Pathol *151:* 671-677, 1997.

15 Charpin C, Garcia S, Bouvier C, Andras L, Bonnier P, Lavaut M-N and Allasia C: CD31/PECAM Automated and quantitative immunochemical assay in breast carcinomas. Am J Clin Pathol *107:* 534-54, 1997.

16 Hauser IA, Riess R, Hausknecht B, Thuringer H and Sterzel RB: Expression of cell adhesion molecules in primary renal disease and renal allograft rejection. Neprol Dial Transplant *12:* 1122-1131, 1997.

17 Jain RK: Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. J Natl Cancer Inst *81*(8): 570-576, 1989.

18 Markov MS: Influence of radiation on biological systems. *In:* Charge and Field Effects in Biosystems II (Allen MJ, ed), New York, Plenum Press, 1990, pp 241-250.

19 Markov MS: Magnetic and electromagnetic fields - a new frontier in clinical biology and medicine. *In:* Proceedings of Millennium International Workshop on Biological Effects of Electromagnetic Fields (Kostarakis P and Stavroulakis P, eds), Athens, Greece, 2000, pp 365-372. ISBN 960-86733-0-5.

20 Folkman J, Hahnfeldt P and Hlatky L: Cancer: looking outside the genome. Nature Reviews: Molecular and Cellular Biology *1:* 76-79, 2001.

21 Hannan CJ, Liang Y, Allison JD and Searle JR: *In vitro* cytotoxicity against human cancer cell lines during pulsed magnetic field exposure. Anticancer Res *14*(4A): 1517-20, 1994.

22 Liang Y, Hannan CJ, Chang BK and Schoenlein PV: Enhanced potency of daunorubicin against multidrug resistant subline KB-ChR-8-5-11 by a pulsed magnetic field. Anticancer Res *17*(3C): 2083-88, 1997.

23 Salvatore JR, Blackinton D, Polk C and Mehta S: Nonionizing electromagnetic radiation: A study of carcinogenic and cancer treatment potential. Reviews on Environmental Health *10*(3-4): 197-207, 1994.

24 Salvatore JR: Nonionizing electromagnetic fields and cancer: A review. Oncology *10*(4): 563-570, 1996.

Received August 8, 2001
Accepted September 5, 2001