J. Steroid Biochem. Molec. Biol. Vol. 43, No. 1–3, pp. 189–195, 1992
Printed in Great Britain

0960-0760/92 $5.00 + 0.00
Pergamon Press Ltd

TAMOXIFEN INDUCED CHANGES IN MCF7 HUMAN BREAST CANCER: *IN VITRO* AND *IN VIVO* STUDIES USING NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND IMAGING

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Summary—The effects of 17β-estradiol versus tamoxifen on the growth and metabolism of MCF7 human breast cancer cells, in culture and in tumors implanted in nude mice, were studied by ^{31}P and ^{13}C nuclear magnetic resonance spectroscopy and by proton magnetic resonance imaging. In culture, the content of the phosphate metabolites including nucleoside triphosphates (NTP), phosphomonoesters, phosphodiesters and inorganic phosphate (Pi) were not affected by tamoxifen treatment. However, in the presence of estrogen the rate of glucose consumption and lactate production via glycolysis (270 and 280 fmol/cell · h, respectively) were twice that of tamoxifen treated cells. Estrogen rescue of tamoxifen treated cells indicated that glycolysis induction occurs at the early stages of the hormonal response. The *in vivo* studies included recording of proton images that provided an accurate measure of tumor size and distribution of tumor cells, necrotic regions and stromal tissue. Tamoxifen caused enhanced necrosis extending from the center of the tumor during the first two days of treatment (12 h to 6 days). This was followed by growth of reparative tissue along with tumor regression. Tamoxifen also modified the content of the phosphate metabolites, increasing markedly ($P < 0.0002$) the ratio of NTP to Pi from 0.41 before treatment to 1.75 9–19 days after treatment. This change was attributed to the enhanced growth of repair tissue. The results provide new information regarding the response of human breast cancer to hormonal treatment and suggest a mechanism for the induction of tumor regression by tamoxifen.

INTRODUCTION

With the development of *in vivo* magnetic resonance imaging (MRI) and spectroscopy (MRS), it is now possible to monitor non-invasive changes in tumor characteristic features including metabolites and metabolic activities. The effects of different modes of cancer treatment on phosphate metabolites were monitored by ^{31}P nuclear magnetic resonance (NMR) studies in a variety of tumors ([1–3] and references cited therein). The effects of ovariectomy and of tamoxifen on the ^{31}P spectra of nitrosomethylurea induced rat mammary tumors were recently described [4–6]. We have previously investigated the effects of estrogen versus tamoxifen on the energetics and glucose metabolism of T47D-clone 11 human breast cancer cells using ^{31}P and ^{13}C NMR measurements [7, 8].

In this report we extend our NMR studies to MCF7 human breast cancer cells grown in culture, *in vitro*, and to tumors implanted in athymic immunodeficient mice, *in vivo*. ^{31}P and ^{13}C NMR were applied to investigate estrogen versus tamoxifen induction of metabolic processes, primarily the energetics and glucose metabolism. Proton images and ^{31}P localized spectroscopy were utilized to monitor estrogen induced growth and tamoxifen treatment and tumor regression. The parallel application of imaging and spectroscopy enabled us to characterize changes in tumor size, the state and distribution of the neoplastic cells, necrotic areas and fibrous tissue and the content of phosphate metabolites during estrogen induced growth and tamoxifen induced remission. The results provide a new insight into the mechanism of action of tamoxifen and may also help to develop NMR methods for assessing reliably the response of breast cancer patients to tamoxifen therapy.

Proceedings of the Fourth International Congress on Hormones and Cancer, Amsterdam, The Netherlands, September 1991.

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EXPERIMENTAL

Cell culture

MCF7 cells were obtained from the laboratory of Professor Lippman (NIH, 1987) and were cultured routinely in Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and mycostatin (10 U/ml). The effects of 17β-estradiol and of tamoxifen were studied using phenol red free DMEM and dextran coated charcoal stripped FCS. Cells for inoculation were detached with 0.03% EDTA in phosphate buffer saline solution and then washed several times in the same buffer solution.

NMR experiments of cells in vitro

For the NMR experiments, cells were grown on polyacrolein-agarose microspheres according to the procedure used previously for T47D human breast cancer cells [9, 10]. We also applied the method of embedding cells in agarose beads previously developed in our laboratory [11]. The cells in the beads were incubated under normal growth conditions for 24 to 48 h before the NMR experiment. When the density of the cells was about 3×10^7 cells per ml beads, 2.5 ml of the beads were transferred to a 10 mm standard NMR tube and perfusion was immediately initiated. The basic features of the perfusion system were described previously [7]. However, two additional elements were introduced: a bubble trap and a second line for gas delivery to the medium container to assure full and constant oxygenation. The perfusion medium was the same as the growth medium. In experiments with 17β-estradiol (30 nM) or tamoxifen (2 μM), phenol red free DMEM and charcoal stripped FCS were used. In ^{13}C NMR studies of glucose metabolism [1-^{13}C]glucose (99%) was added to glucose free medium at a concentration of 5.6 mM.

The NMR measurements were performed with a Bruker AM-500 spectrometer using a quadro-nuclei (^{1}H, ^{31}P, ^{13}C and ^{15}N), software controlled, probe. ^{31}P NMR spectra were recorded at 202.5 MHz applying 90° pulses and 10 s repetition delay and accumulating 180 transients (30 min). ^{13}C spectra were recorded at 125.7 MHz, applying 60° pulses and 6 s repetition delay and accumulating 150 transients (15 min). Composite pulse proton decoupling was continuously applied in both ^{31}P and ^{13}C measurements. The analysis of the data was performed using methods described previously [7, 8].

NMR studies of tumors in vitro [12]

Tumors were implanted in female CD1-NU athymic mice, 6 to 8 weeks old. Before the injection of the cells a pellet of 17β-estradiol, 0.72 mg/pellet (Innovative Research of America) was implanted underneath the back skin. The cells were inoculated into the flank of the mice. Tamoxifen treatment was initiated by replacing the estradiol capsule by a tamoxifen capsule, 5 mg/pellet (Innovative Research of America). For the NMR measurements the mice were anesthetized by i.p. injection of sodium pentobarbital at a dose of 60 μg per g weight of mouse. NMR spectra and images were recorded with a Bruker 4.7/30 Biospec spectrometer. ^{1}H spin echo images with echo times (TE) of 17 and 68 ms and a repetition time of 2500 ms were obtained using a data matrix of 256 × 256 pixels 0.6–1.5 mm slice thickness 3 to 5 cm field of view and 2 to 8 averages. The intensities were mostly proton density weighted in short TE and mostly T_2 weighted in the long TE images. ^{31}P spectra were recorded at 81.1 MHz using the outer volume suppressed image related *in vivo* spectroscopy (OSIRIS) sequence [13] and a ^{31}P–^{1}H double tuned surface coil with a diameter of 11 mm. Spectra were recorded from an 8 × 8 × 8 mm volume assigned in the ^{1}H images [12]. Two sets of experiments were averaged with acquisition parameters consisting of 8 cycles of 64 scans with 10 s delay between cycles and a 2 s delay between scans using 90° adiabatic pulses. The total time for a spectrum recording was 36 min.

At different times of growth and treatment mice were sacrificed and the tumors were removed and fixed in Bouin's solution and then processed for histological sections stained with hematoxylin-eosin and light green. Comparison between ^{1}H images and their corresponding histological sections permitted correlation of the intensities in the images with necrotic regions fibrous areas and proliferating, neoplastic regions. Figure 1 demonstrates the variations in the intensities in T_2 weighted images and their corresponding assignment based on comparison with histological sections.

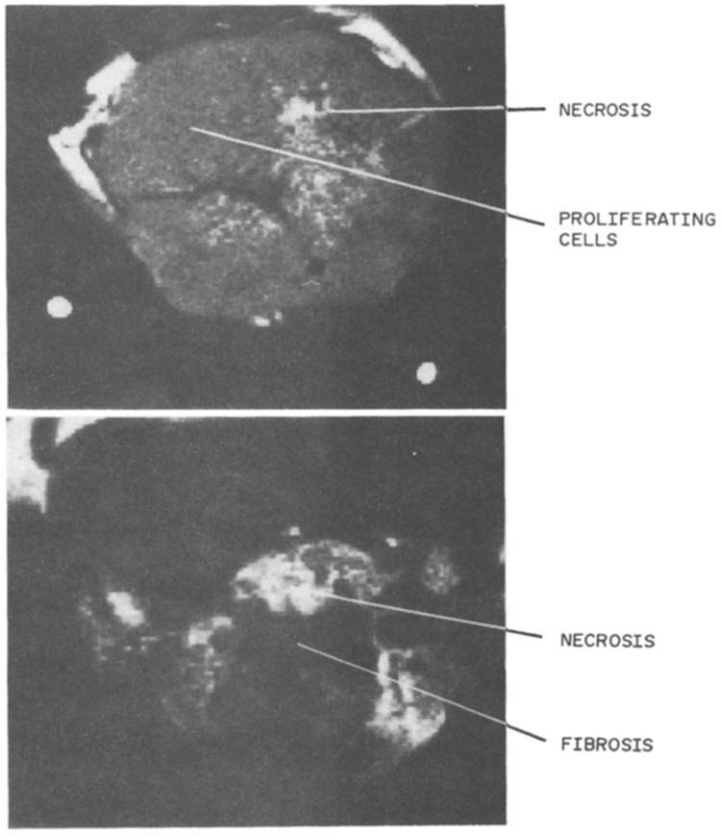

Fig. 1 Proton T_2 weights images of MCF7 tumors. Upper image. tumor recorded 80 days after implantation. Scale: 1 cm in the image is 2.4 mm Lower image: tumor recorded 30 days after tamoxifen treatment Scale 1 cm in the image is 1 5 mm. The images were recorded as described in the Experimental section.

RESULTS AND DISCUSSION

Metabolism of MCF7 cells in vitro

^{31}P NMR spectra of MCF 7 cells grown in agarose beads or on agarose–polyacrolein beads and perfused with growth medium at 37°C revealed the presence of the following intracellular metabolites (Fig. 2): three signals due to the α, β and γ phosphates of nucleoside triphosphates (NTP), a small signal of phosphocreatine (PCr), metabolites involved in phospholipid synthesis, phosphocholine (PC) and phosphoethanolamine (PE), and in phospholipid breakdown, glycerolphosphocholine (GPC) and glycerolphosphoethanolamine (GPE), sugar derivative of uridine disphosphate (UDPG) and inorganic phosphate (Pi). The medium Pi (at pH = 7.5) was very close to the intracellular one, however, a small chemical shift due to a pH difference between the two compartments (0.1 pH unit lower) could be distinguished.

There was no significant difference between the spectra of cells grown on beads or in beads (Fig. 2). Comparison with previous spectra of MCF7 cells maintained during the NMR experiments in agarose threads [14] revealed a similar profile of phosphate metabolites with differences in the relative content of the lipid derived metabolites. This could be due to clonal variations or to differences in the preparation, medium composition and perfusion conditions.

^{13}C NMR measurements using [1-^{13}C]glucose enabled us to monitor glucose consumption as well as lactate production via glycolysis, glutamate synthesis following glucose entrance to the TCA cycle, alanine synthesis and glycogen formation. Figure 3 shows a series of ^{13}C spectra, consecutively recorded after replacing the regular medium by medium containing [1-^{13}C]glucose. The rate of glucose consumption was calculated to be 273 fmol/cell · h. The rate of lactate labeling determined from the gradual increase in the [3-^{13}C]lactate signal was 140 fmol/cell · h, thus suggesting that both aerobic glycolysis and oxidative phosphorylation contribute to energy production. The rate of lactate synthesis (two lactate molecules

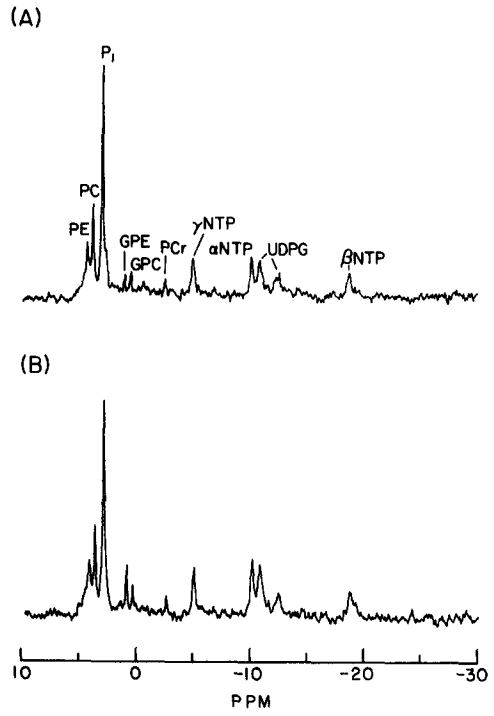

Fig. 2. ^{31}P NMR spectra of MCF7 cells grown in agarose beads (A) or on agarose–polyacrolein beads (B). Cells in or on beads ($\sim 3.10^7$ cells/1 ml beads) were perfused at 37°C with growth medium and recorded as described in the Experimental section. A line broadening of 25 Hz was used.

are synthesized from one glucose molecule, one labeled and one unlabeled) was thus calculated to be 280 fmol/cell · h.

MCF7 cells grown on beads with medium containing stripped serum, no phenol red, and 2 μM tamoxifen exhibited a profile of phosphate metabolites similar to that obtained for cells growing under regular conditions. However the density of the cells was lower since tamoxifen inhibited proliferation. Following estrogen rescue of tamoxifen treated cells no change in the phosphate metabolites was detected. Similar results were obtained previously in ^{31}P studies of T47D human breast cancer cells [8]. However, as in T47D cells [7, 8], under the influence of tamoxifen, the rate of glucose metabolism via glycolysis was 2-fold slower (138 fmol/cell · h) and estrogen rescue of tamoxifen treated cells induced a fast (within 1 h) enhancement in this rate (204 fmol/cell · h). A similar estrogen induction of glucose consumption via glycolysis was also detected in immature rat uteri [15]. These results therefore indicate that one of the early responses to estrogen is concerned with fast stimulation of energy production via glycolysis which in turn is required for enhanced proliferation. Tamoxifen counteracts estrogen induction and slows the rate of energy production and hence proliferation rate.

MCF7 tumors in nude mice

Proton spin echo images and localized ^{31}P spectra of the tumors were recorded during estrogen induced growth. Figure 4 shows T_2

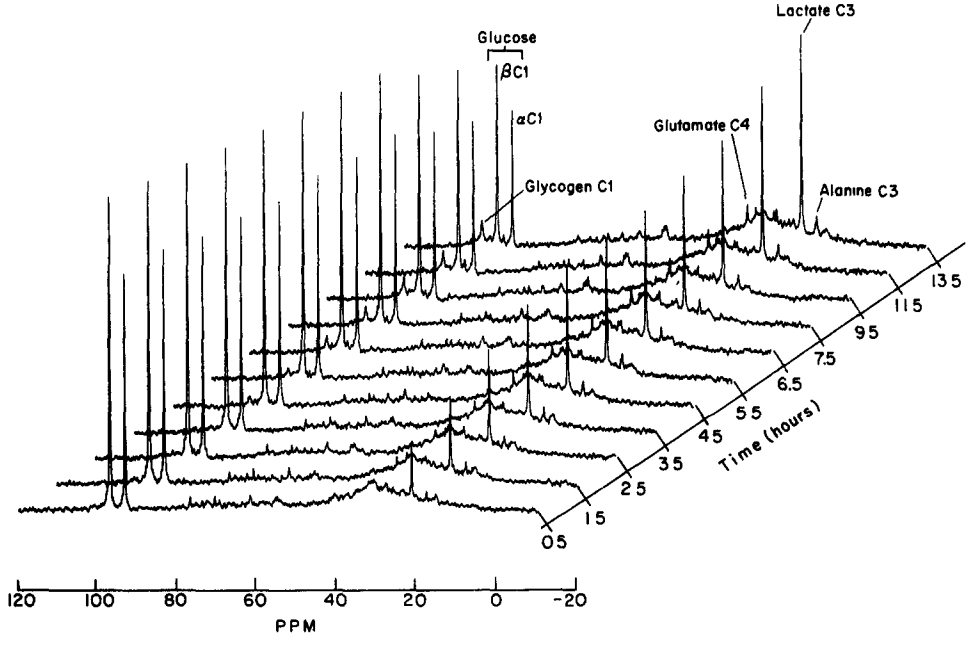

Fig. 3. ^{13}C NMR spectra of MCF7 cells on agarose–polyacrolein beads perfused with growth medium (50 ml) containing [1-^{13}C]glucose (5.6 mM) at 37°C. Each spectrum was accumulated for 15 min as described in the Experimental section.

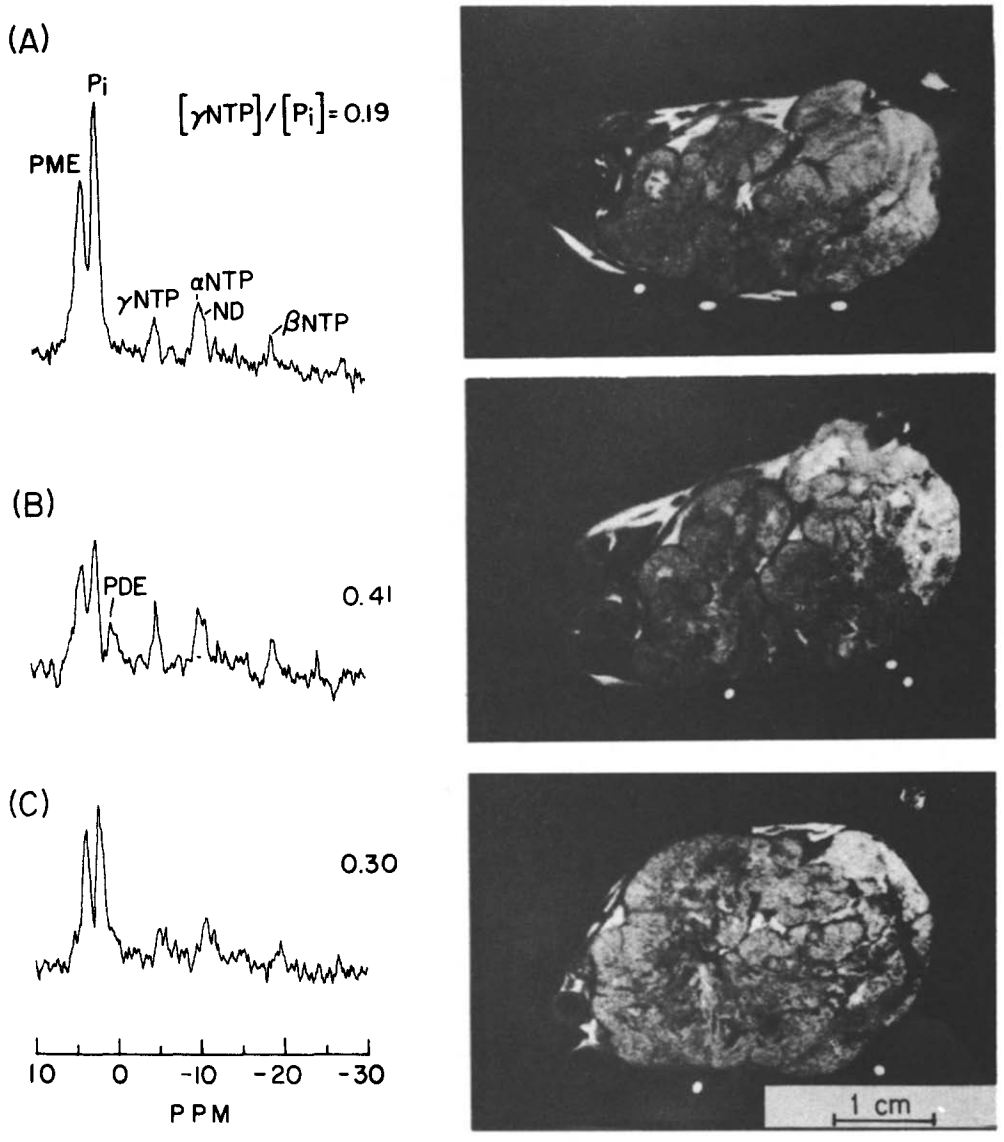

Fig. 4 Proton T_2 weighted images and ^{31}P localized spectra of MCF7 tumor implanted in nude mouse. A, B and C 60, 71 and 86 days, respectively after cell inoculation. Tumor size in cm^3: (A) 7.6; (B) 11 3, and (C) 15.5 Recording conditions of the images and spectra are detailed in the Experimental section

weighted images of a tumor (on the left) and the corresponding ^{31}P spectra. The analysis of the contrasts in these T_2 weighted images was based on comparison with histological sections. It was found [12, 16] that grey areas indicated the presence of viable carcinoma cells, bright, high intensity areas indicated necrotic regions while dark areas revealed fibrous connective tissue. It should be pointed out that this correlation is specific to MCF7 tumors and may not generally hold for other types of tumors (i.e. necrotic regions appear black in T47D tumors in nude mice [16]). Small tumors ($< 1\ cm^3$) are mostly homogenous and exhibit mainly gray areas while large tumors show high heterogeneity (Fig. 4).

The ^{31}P spectra of tumors larger than the localized volume ($0.51\ cm^3$) revealed the presence of PME, Pi, PDE, and NTP. In tumors smaller than the localized volume a substantial signal of PCr appeared which was mainly due to the presence of muscle in this volume. In spectra of tumors the area of the Pi and of the PME signals was higher than that of NTP. The average ratio of NTP/Pi for tumors larger than $1\ cm^3$ was 0.41 ± 0.15 ($n = 14$). This ratio fluctuated for the same tumor (Fig. 4). The pH of the tumors was quite acidic and varied between 6.7

to 7.6 with an average value of 7.00 ± 0.25 ($n = 14$). The PDE signal was usually small and occasionally unresolvable.

Treatment of the tumors with tamoxifen, for 30 days, resulted, in all cases studied ($n = 8$), in a substantial regression to 20–40% of the original volume. The recording of the proton images following the treatment provided unique information regarding the response. Changes in the appearance of the images were detected by 12 to 48 h after tamoxifen administration. The most conspicuous change was the development of large central necrotic regions (Fig. 5). It appeared as if all the central vascularization network collapsed at once with total inhibition of neovascularization. This possibly caused a rapid reduction in blood flow, limiting oxygen and nutrient supply leading to cell death and necrosis.

It has been hypothesized that tumors secrete angiogenic peptides which contribute to tumor neovascularization to provide the blood supply necessary for tumor growth [17, 18]. Tamoxifen is reported to inhibit estrogen mediated secretion of growth factors (e.g. TGFα) [19, 20] which also promote angiogenic activity [21]. It is therefore tempting to propose that the response to tamoxifen *in vivo* is dominated by slowing and inhibiting the production and secretion of angiogenic factors. Although tamoxifen as a cytostatic agent inhibits a host of reactions [22], including the energy production described above, it apparently exerts a cytotoxic effect *in vivo* by enhancing necrosis. This necrosis may result from inhibition of neovascularization or as was recently suggested for estrogen deprived MCF7 tumors [23], from activated apoptosis.

Following the massive necrosis, growth of fibrous tissue was indicated by the appearance of dark, low intensity regions (Fig. 5). Further darkening of the images was observed with the decrease in tumor size suggesting enhanced stromal fibrosis. This change was confirmed by comparison with histological sections of tumors treated with tamoxifen.

The ^{31}P localized spectra of tamoxifen treated tumors showed changes in the profile of the phosphate metabolites, the most prominent being a decrease in the Pi and an increase in NTP. The ratio of NTP to Pi increased from 0.41 ± 0.15 to 1.1 ± 0.7 ($P < 0.02$) 4 to 7 days after treatment and to 1.75 ± 0.66 ($P < 0.0002$) 9 to 19 days after treatment. It was clear from the images and the localization that the signals originated from the tumor. Based on the analy-

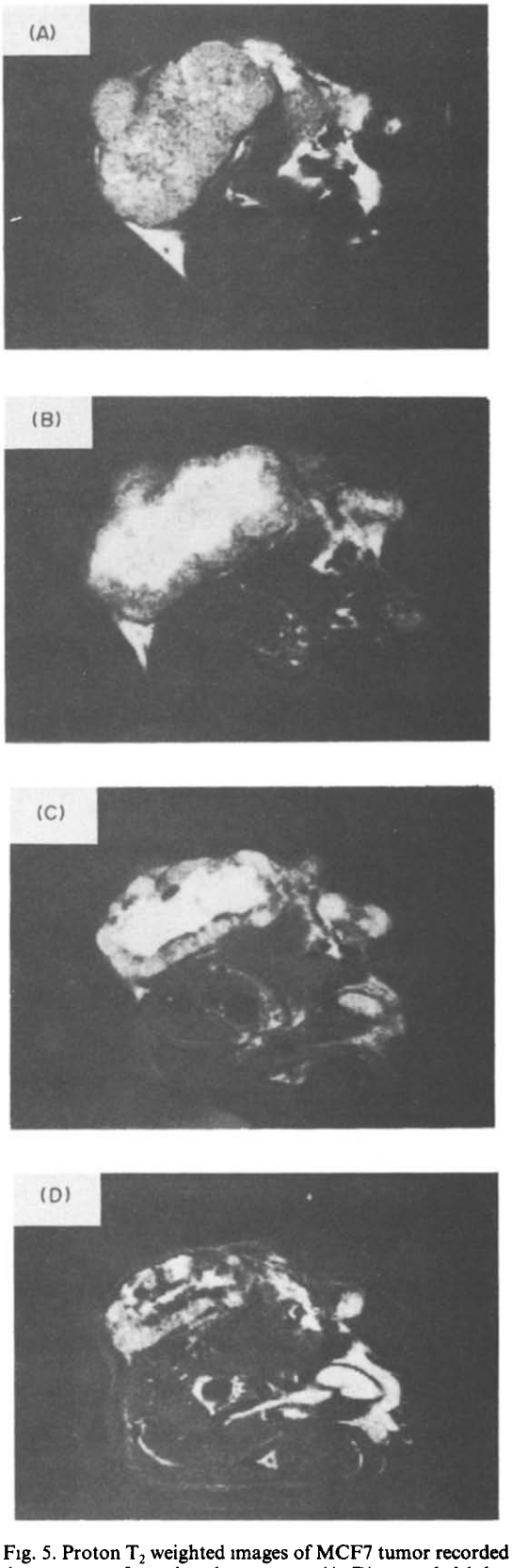

Fig. 5. Proton T_2 weighted images of MCF7 tumor recorded during tamoxifen induced regression. (A–D): recorded 1 day before treatment with tamoxifen and 6, 13 and 27 days after treatment The experimental conditions are described in the Experimental section

sis of the images and histology of the tamoxifen treated tumors we have concluded that the changes in the phosphate metabolites are due to enhanced growth of fibrous reparative tissue. It is not known if the stromal stimulation is a consequence of normal repair process following the rapid necrosis or it is the result of a direct agonistic effect of tamoxifen on the fibroblasts of the host mouse.

In summary we have studied MCF7 human breast cancer both *in vitro* and *in vivo*, utilizing the non-invasive approach afforded by magnetic resonance spectroscopic and imaging techniques. *In vitro* it was possible to demonstrate the inhibiting effect of tamoxifen versus estrogen regarding glucose metabolism and energy production which correlates with the slow down of the growth rate. *In vivo*, short term tamoxifen treatment caused substantial regression by enhancing cell death followed by a repair stromal growth. The processes responsible for the *in vivo* response seem to be associated with tumor vascularization and induced cell death mechanism.

Acknowledgements—This work was supported by the United States Public Health Service National Institute of Health Grant CA-42238, the German Israel Foundation for Research and Development and the Henry and Francoise Glasberg Foundation for Research on Cellular Disease. The authors wish to thank Professor A. M. Kaye from the Hormone Research Department for support and helpful discussions.

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