Changes in Tumor Vascularization after Irradiation, Anthracyclin, or Antiangiogenic Treatment in Nitrosomethyl Ureas-Induced Rat Mammary Tumors

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ABSTRACT

Purpose: Changes in tumor vascularization may be involved in tumor regression after anticancer treatments. We therefore studied the relationship between tumor vascularization and tumor response according to treatment by irradiation (RT), epirubicin (EPI), or antiangiogenic agent TNP-470 in a nitrosomethyl-ureas-induced rat mammary tumor model by measuring the changes in tumor blood flow using high-frequency Power-Doppler sonography.

Experimental Design: Mammary tumors were induced in female Sprague-Dawley rats by a single s.c. injection of nitrosomethyl-ureas. After tumor areas reached 2 cm², the animals received four weekly injections of epirubicin (EPI group), or a single fraction of 18 Gy (RT group), or six injections of TNP-470 within 12 days (TNP group), or both (RT combined with TNP-470, RT+TNP group). Power-Doppler sonography quantification of tumor vascularization (PDI) was performed before and 12 days after initiation of treatment. Tumor shrinkage was later evaluated and compared with the early changes in PDI values.

Results: Compared with the control group, EPI induced an arrest in tumor growth. A similar effect was obtained with TNP-470. There was a decrease in tumor area after RT, but administration of TNP-470 combined with RT did not further enhance this effect. Changes in tumor area paralleled changes in PDI in the EPI group. Furthermore, changes in PDI 7 days after RT were associated with further tumor change in the RT groups, whereas they were independent of the antitumor effect of TNP-470.

Conclusions: Changes in functional tumor vascularization evolution appeared to be closely associated with tumor regression after anticancer treatment.

INTRODUCTION

Vascular changes associated with anticancer treatments, such as radiation therapy or chemotherapy, may be pivotal in subsequent tumor volume decrease or progression (1). Changes in tumor vasculature may occur before tumor regression, as suggested by the temporal changes in tumor blood flow and histological blood vessel quantification reported in a murine tumor system (2) and in humans (3). A potential role of tumor vascularization in tumor volume changes after radio or chemotherapy has not to date been documented. The study of the mechanisms potentially involved in the vascular effects of radio or chemotherapy is difficult. It requires assessment of overall tumor vascularization (caused by heterogeneity of perfusion) with noninvasive methods to avoid tumor damage caused by biopsy. Doppler-sonography depicting the morphology of both peritumor and intratumor vessels has recently been used for its ability to provide accurate data on blood flow velocity from vessels (4), thus providing overall assessment of tumor vascularization. Power Doppler sonography offers many additional advantages, such as high sensitivity, simplicity of use, and repeatability by depicting overall tumor vascularization (5). Quantification of tumor Doppler signals derived from tumor blood flow has been shown to reflect tumor vascularity (6). As recently shown in a rat model of N-nitroso N-methyl urea-induced mammary tumors, the recent development of high-frequency probes makes power Doppler sonography suitable for use in small animals and small tumors and provides a high reproducibility of vascular quantification in vivo (7). Significant correlations between Doppler signal quantification and histological assessment of microvessel density have been reported (2, 8). This technique, which can be applied in the rat to quantify tumor vascularization, allows vascular monitoring during anticancer treatments, such as radiotherapy or antiangiogenic treatment (8, 9).

We used power Doppler sonography to investigate the role of neo-vessels in tumor regression after irradiation or epirubicin-based chemotherapy, by sequentially quantifying early vascular changes in rat mammary tumors. Epirubicin was chosen because it acts as a radiomimetic and is commonly used in the treatment of human breast cancer. The antiangiogenic agent TNP-470 was used as a positive control (10–12).

By focusing specifically on the functional tumor vessels, changes in vascular evolution were found to be closely associated with tumor regression following the different anticancer treatments used.
MATERIALS AND METHODS

Experimental Animal Tumor System. An experimental rat model of mammary tumors was used. Female Sprague-Dawley rats were purchased at 42 days of age and housed three per cage in a temperature-controlled room at 23 °C and maintained under a light schedule of 12-h light/12-h dark. They received water and standard rodent diet. At 48 days of age, each animal received a single s.c. injection of NMU (Sigma Chemical Co.) at a dose of 25 mg/kg body weight in the left side of the abdominal wall (7, 13). Tumor appearance was monitored by weekly manual mammary gland palpation, and tumor area was measured with a caliper. Although tumors arose from several sites, most of the tumors were abdominal.

Anticancer Agents. Tumor area was measured as the product of the two largest dimensions. When tumor area reached 2 cm², animals were randomly assigned to several treatment modalities. Because tumor frequency was observed previously to reach 50–70% (7), rats were ascribed to experimental groups as follows: 10 rats received six s.c. injections of TNP-470 alone (30 mg/kg body weight/day, three times a week for 12 days), generously provided by Takeda Chemical Industries, Ltd. (Osaka, Japan). TNP-470 was suspended in 1% ethanol and dissolved in 0.5% methyl-cellulose-saline solution (TNP group). Forty-four rats were given i.p. injections of epirubicin [2.5 mg/kg/week (Pharmacia and Upjohn Co.) dissolved in saline] for four weeks (EPI group). This dose was found previously to induce a moderate antitumor effect along with acceptable toxicity. Six rats served as a control group and received saline under identical modalities to the other groups. Eighteen rats received a single dose of 18 Gy radiation with a single direct field of electron to tumor (RT group). Irradiation was carried out using a linear accelerator (Elekta Sli, United Kingdom). The energy of the electron beam was determined according to the depth of the target volume (4, 6, or 8 MeV). To deliver the dose to a precise area of the surface and underlying target volume, an additional mask was interposed between the base of the standard collimator and the point of entry of the radiation beam. The mask was made of 2-mm-thick lead plate in which an opening was created to desired dimensions. The opening was defined by first determining the maximum dimensions of the target volume in the plane perpendicular to the radiation beam and adding a margin of 1 cm in each direction. The point of calculation of the delivered dose of radiation was located at the maximum of the dose-dose curve for selected dimensions of the beam at a distance of 100 cm from the skin. This irradiation schedule delivered a dose found previously to induce moderate tumor regression (7). Irradiation was performed under anesthesia using isoflurane (Abbott Co.). Ten rats received a combined treatment (RT-TNP group) comprising two injections of 30 mg of TNP-470 per kg body weight administered 3 days and 1 day before irradiation. Irradiation was followed by four injections of TNP-470 at the same dose and modality as the TNP-470 alone group. Rats in this combined-modality group thus received six injections of TNP-470 within 12 days, a protocol similar to the TNP-470 group. (Fig. 1)

After the study was completed, rats were sacrificed with a pentobarbital injection according to guidelines established by the Institution’s Animal Care and Use Committee. Histopathological assessment of tumor malignancy was randomly performed in each experimental group. Tumors were fixed in Formol-Zinc and embedded in paraffin. Five micrometer sections were prepared and stained with hematoxylin and eosin and subsequently examined by a pathologist. All treated tumors were macroscopically similar and histologically were well differentiated adenocarcinoma of papillary type.

Definition of Tumor Sensitivity to Experimental Agents. To compare changes in PDI to the profile of tumor growth after application of the experimental agents, each group was subdivided into sensitive and resistant tumors. In the epirubicin and TNP-470 groups, tumors were split into two subgroups: (a) a sensitive group in which tumor area was decreased at day 28 compared with day 1; and (b) a resistant group in which tumor area at day 28 was greater than at baseline. Tumors in the irradiated group were considered to be sensitive to irradiation when tumor area (assessed at day 28) decreased by >50% of baseline area. When the tumor area had decreased by <50% at day 28, the tumor was defined as resistant. A similar subdivision was applied to the combined RT-TNP group.

2 The abbreviations used are: PDI, power-Doppler index; VEGF, vascular endothelial growth factor.
Ultrasound Examination. Gray scale and power Doppler sonography were performed under anesthesia as already described (7). The duration of ultrasonography examination was limited to 30 min to prevent hypothermia because of anesthesia. Tumors were scanned with a 7–10 MHz linear probe (LA 523) using a Technos scanner (ESAOTE, Italy). Tumor compression was kept to a minimum, demonstrated by the persistence of gel between the probe and the tumor on the monitor to avoid tumor flow changes.

We analyzed only abdominal tumors and not tumors that arose from other sites to maintain similar conditions of Doppler examination between animals. Gain and velocity scale were the same throughout the experiment for all tumors. The five best power Doppler sonography images of the tumor corresponding to the subjectively determined highest Doppler signal were obtained from different imaging planes. Images were stored digitally and then transferred to a computer for further quantitative analysis of vascularization.

Quantification and Chronology of Tumor Vascularization. The PDI of tumor vascularization was quantified using dedicated software for color pixel quantification developed from a Matlab program. This software allows determination of the number of colored pixels in a manually drawn region of interest corresponding to the whole tumor, regardless of signal intensity. The PDI was calculated as relative number of pixels in the ultrasound image displaying a power Doppler signal, i.e., the number of colored pixels/number of pixels in the region of interest. The PDI was calculated in each tumor using the mean value of five stored images of a tumor to reflect the mean PDI of the tumor. Measurements were performed 1 h before treatment (day 1) in control, TNP-470, and epirubicin groups. The first measurement was performed 3 days before irradiation in the irradiated group and 1 h before the first injection of TNP-470 in the RT-TNP group. A second measurement was carried out 12 days after the first (Fig. 1). This time was selected as long enough to allow vascular change to be observed and short enough to prevent any detectable change in tumor size.

Statistical Analysis. The significance of differences in tumor growth among groups was evaluated using a nonparametric Mann-Whitney U test. Baseline values of tumor PDI were first compared between groups using a nonparametric Mann-Whitney U test. Changes in tumor growth following experimental conditions were compared using the Mann-Whitney U test. Nonparametric tests were used for statistics because q-q plots of the growth data demonstrated a non-Gaussian distribution. Results were considered statistically significant at \( P = 0.05 \).

RESULTS

Effects of Treatment Modalities on Mammary Tumor Growth. Three anticancer treatment modalities were compared. There was continuous tumor growth in the saline-injected group, whereas tumor growth was affected in all other groups. The anticancer agents used had significant toxicity; two rats died in the TNP alone group. One rat died after the fifth injection of TNP-470 and could not be evaluated for tumor growth or PDI changes. The second rat from this group died at day 14 after the sixth injection and was evaluated for PDI changes induced by TNP-470 but not for tumor growth at days 21 and 28. Another rat in the RT-TNP group died after the sixth injection of TNP-470 and was evaluated for PDI changes induced by this combined treatment but not for tumor growth at days 21 and 28. There was no apparent cause of death at autopsy for these three animals. In the epirubicin-treated group, four animals died before day 28. Three rats had purulent peritoneal effusion. One rat died with cachexia and had liver damage. Detailed growth curves are presented in Fig. 2 for mammary tumors treated with saline, epirubicin, TNP-470, and irradiation alone or combined with TNP-470. Top panel, each point represents the mean of tumor area variation in each group according to time. Bars, SD. Middle panel, number of tumors evaluated at each time point. Bottom panel, statistical analysis (Mann-Whitney U test was used for comparison.).

Early Assessment of Vascular Changes Induced by Treatment Modalities. Mean baseline values of the PDI calculated before treatment were not statistically different between control, EPI, TNP-470, RT, and RT-TNP groups (13, 11, 15, 12, and 12%, respectively; \( P = \text{NS} \)).

Fig. 3 shows PDI changes according to treatment. The PDI was increased in both saline (+56%) and epirubicin-treated tumors (+22%; \( P = \text{NS} \)), whereas it was significantly reduced after either six injections of TNP-470 (−44%) or irradiation.
and the combined RT+TNP-470 treatment (−53%, 
P < 0.05). Coadministration of TNP-470 with RT did not significantly increase the antivascular effects of irradiation. Early changes in PDI were not significantly different between the RT, TNP-470, and combined RT+TNP-470 groups.

Tumor Vascular Changes and Sensitivity of Tumors to Anticancer Agents. There was an association between PDI changes at day 12 and tumor growth according to treatment modality. When tumors were separated into two groups according to responsiveness to treatment, three PDI profiles emerged. In the epirubicin group (Fig. 4A), PDI at day 12 was greater in tumors that proved to be epirubicin resistant than in epirubicin-sensitive tumors, although tumor area was greater in the epirubicin-resistant group than in the epirubicin-sensitive group at day 12. PDI changes (assessed at day 12) were not significantly different in control and epirubicin-resistant
tumors and were significantly greater than in epirubicin-sensitive tumors.

In irradiated tumors (RT alone and RT + TNP-470 groups), both sensitive and resistant tumors had lower PDI than controls (Fig. 4, C and D). The decrease in PDI was significantly greater in radiosensitive than radioresistant tumors. Differences in PDI appeared earlier than differences in tumor shrinkage, irrespective of the concomitant administration of TNP-470. This pattern was not found in the TNP-470 alone treated group. In the latter (TNP-470), tumor growth at day 28 appeared not to be dependent on tumor vascularization changes measured at day 12. In both TNP-470 resistant and sensitive tumors, PDI was lower than in controls, but it was not different between resistant and sensitive tumors (Fig. 4B).

**DISCUSSION**

Using Doppler sonographic evaluation to quantitate functional tumor vascularization in rat mammary tumors, we found changes in PDI to be closely associated with tumor regression following anticancer treatments. The timing and intensity of changes in PDI seemed to differ between treatments, suggesting that various mechanisms may be involved.

TNP-470 administered in six sequential injections provided an antitumor effect, which was associated with a 30% decrease in PDI. Not all mammary tumors regressed after TNP-470 administration. However, PDI decreased in both TNP-470 sensitive and resistant groups. Furthermore, changes in PDI intensity occurred independently of early (day 12) and late (day 28)
tumor regression, thus suggesting that the antiangiogenic action of TNP-470 may not be sufficient to induce antitumor effects. It has been demonstrated that the efficacy of antiangiogenic agents cannot be visualized simply by changes in microvessel density during treatment (14). Although the functional status of the tumor vasculature may provide additional information, we found no correlation between tumor shrinkage and vascular collapse after administration of TNP-470. Mechanisms of TNP-470 action may involve inhibition of neoangiogenesis through inhibition of VEGF production (10). Tumor growth inhibition may result from suppression of cell cycle progression from the G1 to the S phase (15). Although investigators have found a decrease in vessel density after TNP-470 treatment of xenograft brain tumors (16, 17) and renal carcinoma (9), Lund et al. (18) showed no significant difference in vascular density (assessed by histological Chalkley count) between control and treated tumors. TNP-470 may preserve existing vessels, allowing anticancer agents to continue to reach tumor cells (15, 19).

Epirubicin-based chemotherapy (at the dose used) showed an antitumor effect similar to that of TNP-470 but did not induce significant decreases in PDI when compared with TNP-470-treated tumors. The PDI did not change concomitantly with tumor shrinkage in epirubicin-sensitive tumors. In contrast, PDI increased in resistant growing tumors. Yamamoto et al. (20) reported no direct antiangiogenic effect attributable to doxorubicin. In our model, chemosensitive tumors showed a decrease in PDI similar to that found in TNP-470-treated tumors. Whether epirubicin induces a reduction in angiogenic molecules, such as VEGF or basic fibroblast growth factor, after tumor cell destruction, as already suggested (21), has not yet been demonstrated.

Irradiation led to a decrease in PDI 7 days after administration of a single 18 Gy fraction similar to the decrease found with TNP-470, although the antitumor effect was greater. Addition of TNP-470 to irradiation did not lead to a significant increase in PDI changes and did not enhance the antitumor effect observed with irradiation alone, suggesting that TNP-470 has no additional antitumor effect. This comes as no surprise because irradiation may be toxic to both endothelial and epithelial mammary tumor cells, therefore damaging cellular targets of TNP-470. This is in agreement with results reported previously by Murata et al. (22) using single-fraction irradiation combined with TNP-470. They found no synergistic effect of adding TNP-470 to single dose or fractionated irradiation, while also finding an antitumor effect for TNP-470 alone.

Our study indicates that vascular damage after irradiation may be an early indicator of tumor radiosensitivity. Changes in PDI seem to precede late tumor shrinkage after irradiation (1, 2). Although the area of all irradiated tumors had decreased at day 12 (~35%), tumors with an early reduction in PDI compared with baseline had marked shrinkage 4 weeks after irradiation. Growth of other tumors with stable PDI at day 12 resumed later. It is currently thought that irradiation induces tumor and vascular cell damage leading to transitory nonfunctional vessels (1, 18). Synthesis of VEGF by surviving cells after irradiation leads to increased microcirculation and restores blood flow, resulting in early tumor regrowth (23, 24). Moreover, induction of VEGF expression by irradiation contributes to the protection of tumor blood vessels from radiation-mediated cytotoxicity and thereby to tumor radioresistance (25, 26).

In conclusion, changes in PDI appear to be an early indicator of the antitumor activity of irradiation. Such changes evince concomitantly with the antitumor effect of epirubicin and do not seem to be correlated with antitumor action of TNP-470.

We are currently investigating whether changes in tumor vascularization induced by preoperative chemotherapy in locally advanced breast cancer indicate tumor sensitivity to the anticancer agents used.

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REFERENCES


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