Effect of small-sized liposomal Adriamycin administered by various routes on a metastatic breast cancer model

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Abstract

The antitumor effects of small-sized liposomal Adriamycin (LADR) administered by various routes were investigated in rabbits bearing well-developed VX2 tumors in the mammary gland. Rabbits received s.c. or i.v., or s.c. combined with i.v., injections of LADR 6 weeks after tumor implantation. The i.v. route showed a significant inhibitory effect on breast tumors and distant metastases. In comparison, metastases in axillary and mediastinal lymph nodes were more efficiently inhibited after s.c. injection. LADR administered concurrently by both the i.v. and s.c. routes produced satisfactory therapeutic activities on both primary breast tumors and metastases in local-regional lymph nodes, lungs and liver, as shown by slowed growth rates, decreased mRNA expression of proliferating cell nuclear antigen, and extensive necrosis and apoptosis of tumor cells. It is concluded that small-sized LADR administered s.c. provides reliable efficacy on lymphatic metastases of breast cancer and that the addition of treatment by the s.c. route to that by the conventional i.v. route can be recommended as a promising procedure to enhance chemotherapeutic effects in patients with metastatic breast cancer.

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Introduction

Adriamycin (ADR) plays an important role in the treatment of breast cancer. In addition to being a mainstay of therapy in the adjuvant and neoadjuvant settings, ADR is widely used in chemotherapy regimens for patients with metastatic breast cancer (MBC) (Sledge et al. 2003). ADR-containing regimens possess significant advantages in survival over non-ADR-containing regimens (Winer et al. 2001). Despite its excellent antitumor activity, ADR has a relatively low therapeutic index, and its clinical utility is limited due to acute and chronic toxicities such as myelosuppression, immunosuppression and dose-cumulative cardiotoxicity. A retrospective analysis of three phase III trials of patients treated with ADR in combination with other cytotoxic agents or radiation therapy demonstrated that ADR-associated cardiac events may occur more frequently and at lower cumulative doses than previously reported (Swain et al. 2003). Encapsulation of ADR into liposomes may minimize the side effects and enhance the antitumor efficacy by altering its pharmacokinetics and biodistribution pattern. There are two liposomal ADR (LADR) formulations currently under investigation for the treatment of breast cancer: LADR (Myocet/D-99), and pegylated LADR (Caelyx/Doxil). Myocet/D-99 in combination with cyclophosphamide is approved in the European Union as a first-line treatment for MBC. Growing evidence supports the use of Myocet or Caelyx as a substitute for ADR to increase the therapeutic index by maintaining the antitumor efficacy while improving the safety profile (Batist et al. 2001, Harris et al. 2002, Sagra 2003, Pavlick et al. 2004). Regardless of their predominant uptake by the mononuclear phagocyte system after i.v. administration (Bao et al.)
2004), liposomes show a selective accumulation in lymph nodes after local parenteral administration such as s.c., intratumoral, i.m., submucosal and i.p. injection (Oussoren & Storm 2001). This property has been used recently for the delivery of therapeutic and imaging agents to metastasized lymph nodes (Akamo et al. 1997, Kiyokawa et al. 1999, Harrington et al. 2000). Liposome size is one of the most important factors that determines the rate and extent of liposome drainage from the injection site. Small-sized liposomes, 100 nm or so in diameter, seem to achieve relatively high lymphatic uptake (Ikomi et al. 1999, Moghimi & Bonnemain 1999, Oussoren & Storm 1999, 2001, Corvo et al. 2000, Hamaguchi et al. 2003).

The main purpose of the present study was to gain more insight into the potential value for the treatment of patients with MBC of small-sized liposomes given by the i.v. or parenteral routes. LADR of 120 nm in diameter were prepared and a late-stage breast cancer model was established. LADR was administered by various routes and the antitumor effects were assessed in terms of tumor growth inhibition, proliferating cell nuclear antigen (PCNA) expression, necrosis and apoptosis.

Materials and methods
LADR preparation and characterization

Large unilamellar vesicles were prepared by the extrusion method described by Hope et al. (1993). One hundred milliliters of chloroform containing 1.0 mmol (760.9 mg) of egg phosphatidylcholine and 0.82 mmol (316.4 mg) of cholesterol were dried under a stream of nitrogen gas to form a homogeneous lipid film. The trace amount of solvent was then removed under vacuum overnight. The lipid film was hydrated in a 20 ml of low pH citrate buffer (pH 4.0, 300 mM) by vortex mixing. The resulting multilamellar vesicles were frozen/thawed (liquid nitrogen/55°C) five times and extruded ten times at 55°C through two stacked 100 nm polycarbonate filters (Nuclepore, Pleasanton, CA, USA) employing an extrusion device (Lipex Biomembranes, Inc., Vancouver, BC, Canada). ADR was encapsulated by the pH gradient-driven method as described previously by Cullis et al. (1997). The final product had the appearance of a reddish, semitransparent, colloidal solution and was essentially similar to the commercial product Myocet. Under transmission electron microscopy, LADR showed a global, regular contour with homogeneous size and distribution. The mean diameter was 120 nm, as measured by a laser diffraction particle size analyzer. The drug-to-lipid ratio was 0.25 to 1. The drug-embedding ratio was more than 98%. Just before use, normal saline was added to adjust the final concentration of total ADR to 1.0 mg/ml.

Animal model

Forty female New Zealand White rabbits weighing from 2.0 to 2.3 kg were provided by and bred in the Laboratory Animal Center of our University under routine conditions according to the Institute’s ethical and environmental guidelines. The breast cancer model used was as described previously (Chen et al. 2004a,b). Briefly, 1 ml of suspension containing 10⁷ VX2 carcinoma cells (Funabashi Farm Co., Ltd, Osaka, Japan) was injected into the thigh muscle of a carrier rabbit. One week later, the induced tumor was excised under sterile conditions and soaked in 20 ml Hanks’ balanced salt solution. The tissue was sheared to a block of approximately 5 × 5 × 5 mm and then diced into small masses of 0.5–1.0 mm in diameter. The final suspensions were extracted into a 20 ml syringe and injected (0.5 ml) into the mammary gland underneath the right second nipple.

Treatment

Treatment was carried out 6 weeks after tumor implantation. A total of 33 animals was used; seven animals of the original 40 were not included since they developed disseminated breast cancer metastases and did not survive. Animals were divided into four groups. Group A was the control and received normal saline injected s.c. Group B received LADR s.c. adjacent to the breast tumor. Group C received LADR via the auricular vein. Group D received LADR injected s.c. combined with i.v. The dose of LADR in each administration was 1 mg/kg. In group D, one half of the LADR was administered by the s.c. route and the other half by i.v. injection. Treatment was repeated every 48 h. Rabbits were killed 48 h after the third treatment. The longest (a) and shortest (b) diameters of breast tumors and axillary nodes were measured before and after treatment. The volume was calculated as (a × b²)/2. The growth ratio was calculated as ((Volume after treatment/Volume before treatment) – 1) × 100%.

The breast tumors, axillary lymph nodes and all of the metastases pathologically detected in distant organs were collected. The central necrotic tissues in tumors were dissected. Each specimen was divided into three parts for histological, RT-PCR and TUNEL tests.
PCNA mRNA analysis

Using Trizol reagent (Gibco BRL), RNA was extracted from 100 mg specimens, 2 μg of which were used for the RT-PCR reaction. Rabbit PCNA-specific primers were designed based on the published literature (Mandava et al. 2002): forward primer, 5′-TAG-TGGCCACAACTCGCCACCATT-3′ and reverse primer, 5′-GGTCAGTTGCCGTCGACGGGTA-3′ (223 bp product).  β-actin-specific primer sequences were: forward primer, 5′-CTTCGCGGCGACTG-3′ and reverse primer, 5′-GAAGGTCTCAAACTG-3′ (830 bp product). The thermocycling conditions were 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, at 55 °C for 35 s and at 72 °C for 1 min, and a final extension at 72 °C for 7 min. After agarose gel electrophoresis, the densitometry values of PCR-amplified productions were measured by an automated gel image analysis system. RT-PCR values are represented as a ratio of the density values of PCNA divided by those of the internal standard β-actin.

Histological examination

The specimens were fixed in 10% neutrally buffered formalin and embedded in paraffin. The sections were cut at 5 μm and mounted on glass slides overnight at room temperature and then subjected to hematoxylin and eosin staining. The necrotic area in each section was analyzed morphometrically with image processing software (NIH Scion Image, Frederick, MA, USA). The degree of necrosis was determined as the percentage of the necrotic area in the total tumor area.

TUNEL staining

Apoptotic cells were stained with an apoptosis detection kit (ApopTag(R) S7101; Intergen Company, Purchase, NY, USA). The ends of the DNA fragments were labeled by incubation with digoxigenin-labeled dUTP and terminal deoxynucleotidyl transferase. After end-labeling, the specimens were incubated with horseradish peroxidase-conjugated anti-digoxigenin monoclonal antibody and developed with diaminobenzidine.

Table 1 Distribution of animals and tumor metastases in treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Metastases in ALN</th>
<th>Metastases in MLN</th>
<th>Metastases in lungs</th>
<th>Metastases in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7</td>
<td>7</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>8</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>8</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

ALN, axillary lymph node; MLN, mediastinal lymph node.

An apoptotic index (AI), defined as the ratio of apoptotic cells to normal cells, was determined by counting the percentage of TUNEL-positive cells against total nucleated cells in five different sectors per tissue section.

Statistical analysis

The significance of differences was determined with one-way ANOVA followed by a Fisher’s LSD test. P<0.05 was considered to indicate statistical significance.

Results

Animals and metastases

About 1 week after tumor inoculation, palpable entities were induced at the injected site in 31 rabbits. Metastases were present in axillary nodes in 28, mediastinal nodes in 12, lungs in 14, and livers in 3 rabbits. The distribution of the animals and metastases in each group is listed in Table 1.

Tumor or node growth inhibition

The detailed results for the volumes of breast tumors and axillary nodes before and after treatment are shown in Table 2. No significant differences in volumes were found among groups before treatment (P>0.05). After treatment, the volume of breast tumors enlarged by 0.58- and 0.47-fold respectively in groups A and B (P=0.263). Compared with group A, the growth rate was reduced by 15.8 and 12.7% respectively in groups C and D (P=0.009 and P=0.036 respectively). Although the slowest tumor growth was found in group C, this was not significantly different from group D (P=0.589). The volume of axillary lymph nodes enlarged by 2.70-fold in group A; in contrast, the growth was reduced by 58.1% in group B with a significant difference from group C (P=0.001), but not from group D (P=0.148). Group D showed a slower node growth than group C (P=0.049).
Table 2 Volumes of the breast tumors and axillary nodes before and after treatment (means ± s.d.)

| Group | Number | Volume of breast tumors (cm³) | | | | Volume of axillary nodes (cm³) | | | |
|-------|--------|-------------------------------|---|---|---|---|---|---|
|       | Before treatment | After treatment | GR (%) | Before treatment | After treatment | GR (%) | | | |
| A     | 7      | 250.01 ± 24.72 | 393.35 ± 45.72 | 58 | 0.05 ± 0.005 | 0.19 ± 0.04 | 270 | | | |
| B     | 7      | 265.00 ± 29.59 | 386.31 ± 28.23 | 47 | 0.05 ± 0.005 | 0.08 ± 0.02 | 55 | | | |
| C     | 9      | 271.30 ± 38.39 | 355.14 ± 37.54 | 33 | 0.05 ± 0.004 | 0.13 ± 0.03 | 151 | | | |
| D     | 8      | 273.79 ± 32.79 | 375.31 ± 51.45 | 38 | 0.05 ± 0.005 | 0.10 ± 0.03 | 96 | | | |

GR, growth ratio.

PCNA mRNA expression

Table 3 shows the relative values of PCNA mRNA expression in breast tumors, axillary and mediastinal nodes, and distant metastases after treatment. All specimens had detectable mRNA for PCNA. Compared with group A, PCNA expression in breast tumors was decreased by 24.5 and 16.3% respectively in groups C and D (P = 0.001 and P = 0.022 respectively), whereas it was not significantly altered in group B (P = 0.425). The difference between groups C and D was not marked (P = 0.203). The increasing order of PCNA expressions in axillary nodes was as follows: group B < group D < group C < group A. The expression in group B was significantly lower than in groups A or C (P < 0.001 and P = 0.003 respectively), but not than in group D (P = 0.103). The order of PCNA expressions in mediastinal nodes was similar to axillary nodes. The data were not analyzed because of the small number of involved mediastinal nodes. The lowest PCNA level in distant metastases was detected in group D with a significant difference from group A (P = 0.011), but not from groups B or C (P = 0.100 and P = 0.866 respectively).

Tumor necrosis

Table 4 shows the degrees of tumor necrosis in breast tumors, axillary and mediastinal nodes, and distant metastases after treatment. Under microscopy, tumor cells were undifferentiated with a high degree of pleomorphism and arranged in broad sheets, cords, or small acinar-like structures. In the specimens collected from animals of group A, only small areas of necrotic cells were observed in the center of neoplastic cell nests. In comparison, the mean percentage of necrotic area in axillary lymph nodes was increased by 22.1-fold in group B, significantly higher than that in groups C or D (P = 0.001 and P = 0.016 respectively). The mean percentages of tumor necrosis in breast tumors and distant metastases were increased by 12.4- and 14.2-fold respectively in group C; this is significantly higher than the values in group D (P < 0.001 and P = 0.022 respectively). On the contrary, the necrosis in axillary lymph nodes was more severe in group D than in group C (P < 0.001).

Tumor apoptosis

Table 5 shows the detailed results for the average AI of tumor cells in breast tumors, axillary and mediastinal lymph nodes, and distant metastases after treatment. Compared with group A, apoptotic VX2 cells in breast tumors were increased by 1.8- and 2.0-fold respectively in groups C and D (P = 0.001 for both), whereas they were not significantly altered in group B (P = 0.900). The difference between groups C and D was not significant (P = 0.434). The AI in axillary nodes was increased by 3.2-fold in group B with significant differences from groups A and C (P < 0.001 and P = 0.005 respectively), but not from group D (P = 0.542). The AI in distant metastases was increased...
by 1.3-fold in group B ($P = 0.039$) and further increased by 1.7- and 1.5-fold respectively in groups C and D, with significant differences from group B ($P = 0.005$ and $P = 0.026$ respectively).

**Discussion**

Breast cancer is by far the most frequent cancer in women throughout the world and its incidence continues to increase (Parkin *et al*. 1999). Regional lymph nodes are the most common site, and the lungs, liver and bone are the organs most susceptible to the dissemination of advanced breast cancer. Effective control of metastatic tumor cells lurking in the local and distant tissues is essential if therapeutic outcomes are to be improved.

Recent progress in liposome research brings the promise that it may be now possible to attain this goal. A number of randomized clinical trials confirm that Myocet or Caelyx have comparable efficacy in the treatment of MBC with a significantly lower incidence of cardiotoxicity and significantly fewer cardiac events than aqueous ADR (Batist *et al*. 2001, Harris *et al*. 2002, Sagra 2003, Pavlick *et al*. 2004). In addition, recent studies on pharmacokinetics and body distribution have demonstrated the superiority of LADR in the targeted delivery of drugs to specific sites. It was revealed that circulating liposomes are mainly retained by the fixed macrophages in the liver (Kupffer cells), lungs (alveolar interstitial macrophages), spleen and marrow (Ahsan *et al*. 2002, Bao *et al*. 2004). After i.v. administration in MBC patients, Myocet may slow blood clearance and reduce the distribution volume of the encapsulated agents in comparison with the aqueous formulation (Swenson *et al*. 2003).

Due to the peculiar nature and anatomy of the lymphatic system, localization of anticancer agents in tumor-involved lymph nodes is hard to obtain with systemic chemotherapy. Many attempts have been thus directed towards the use of colloidal carriers and local interstitial administration to achieve lymphatic chemotherapy. Among the colloidal carriers proposed for lymphatic targeting, activated carbon particles and liposomes have aroused the most interest. In a previous study, we confirmed that s.c. injection of a carboplatin-activated carbon suspension could effectively and continuously improve the drug concentrations in axillary lymph nodes in patients with breast cancer, in comparison with i.v. injection of carboplatin solution (Chen *et al*. 2004b).


Over recent years, much attention has been paid to the relationship between lymphatic targeting and liposome size. It was demonstrated that uptake of small neutral liposomes in regional lymph nodes after s.c. injection might be up to 30- to 40-fold higher than......

<table>
<thead>
<tr>
<th>Group</th>
<th>Breast tumors</th>
<th>Axillary nodes</th>
<th>Mediastinal nodes</th>
<th>Distant metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>NP</td>
<td>Number</td>
<td>NP</td>
</tr>
<tr>
<td>A</td>
<td>7</td>
<td>3.43 ± 0.53</td>
<td>7</td>
<td>2.71 ± 0.50</td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>3.14 ± 0.38</td>
<td>7</td>
<td>62.57 ± 7.09</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>45.89 ± 6.41</td>
<td>8</td>
<td>39.50 ± 5.71</td>
</tr>
<tr>
<td>D</td>
<td>8</td>
<td>33.88 ± 4.83</td>
<td>6</td>
<td>53.67 ± 6.88</td>
</tr>
</tbody>
</table>

**Table 4** The percentages of tumor necrosis (NP) in breast tumors, axillary and mediastinal nodes and distant metastases after treatment (% ± s.d.)

<table>
<thead>
<tr>
<th>Group</th>
<th>Breast tumors</th>
<th>Axillary nodes</th>
<th>Mediastinal nodes</th>
<th>Distant metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Al</td>
<td>Number</td>
<td>Al</td>
</tr>
<tr>
<td>A</td>
<td>7</td>
<td>5.97 ± 0.84</td>
<td>7</td>
<td>5.16 ± 0.89</td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>5.74 ± 1.76</td>
<td>7</td>
<td>21.73 ± 3.45</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>16.74 ± 4.73</td>
<td>8</td>
<td>16.29 ± 2.85</td>
</tr>
<tr>
<td>D</td>
<td>8</td>
<td>18.04 ± 4.21</td>
<td>6</td>
<td>20.57 ± 4.79</td>
</tr>
</tbody>
</table>

**Table 5** The Al in breast tumors, axillary and mediastinal nodes and distant metastases after treatment (% ± s.d.)
uptake in the liver and spleen (Oussoren et al. 1997). An excellent review presented by Oussoren & Storm (2001) reported that the decisive factors influencing lymphatic absorption of interstitially administered liposomes are liposome size and the anatomical site of injection, and that phagocytosis by macrophages is one of the most important mechanisms for uptake of liposomes by lymph nodes. Colloidal particles with a diameter of more than 520 nm administered s.c. are less likely to appear in lymphatic fluid (Ikomi et al. 1999). In comparison, liposomes about 100 nm in diameter may also be taken up by cells other than macrophages, such as endothelial cells lining the lymph node sinuses, which are capable of pinocytosis of macromolecules and tiny particles, resulting in more opportunities for lymphatic targeting (Oussoren & Storm 1999). It was found that the degree of lymphatic absorption can reach levels up to 70% of the injected dose for small liposomes (Oussoren et al. 1997). Other authors argued that the extent of lymphatic targeting is partly related to the thin-walled and fenestrated lymphatic microvessel, which can be easily penetrated by macromolecular agents and particles (Moghimi & Bonnemain 1999). Corvo et al. (2000) entrapped superoxide dismutase in liposomes and monitored its fate and therapeutic activity in a chronic arthritis inflammation model after s.c. administration. Their data showed that liposomes about 110 nm in diameter resulted in a 17-fold higher uptake and substantially higher activity in the inflamed foot than those about 450 nm in diameter. Similar phenomena were also observed in the treatment of cervical lymph node metastases in a rabbit VX7 tongue tumor model with liposomes of 94.1 nm in diameter (Hamaguchi et al. 2003). These important findings provide the basis for our selection of small-sized LADR in the present study.

The selection of the breast cancer model was based on our previous study, during which we monitored the growth and metastasis of VX2 carcinoma embedded in the mammary gland of rabbits by palpation, X-ray, computed tomography, magnetic resonance imaging and [18F]fluorodeoxyglucose positron emission tomography. VX2 tumors show a rapid growth and high potential for metastasizing to the regional lymph nodes and distant sites such as the lungs, liver and bone. More importantly, metastasis reflected quite closely the metastatic pattern of human breast cancer: the axillary lymph nodes are the most common path and the lungs are the organ most susceptible to metastasis.

Data from studies of i.v. administration suggest that this approach may represent a useful means to treat breast tumors and their distant metastases by inhibiting proliferation while inducing tumor cell apoptosis or necrosis. In addition, the i.v. route showed some antitumor activity against metastasized lymph nodes in the axilla and mediastinum. To our knowledge, there are three likely mechanisms to explain the phenomena. First, it is well known that both prolonging the duration and elevating the concentration of ADR enhances its cytotoxic efficacy (Rupniak et al. 1983). Swenson et al. (2003) reported that compared with conventional ADR, Myocet substantially slowed the blood clearance and improved the plasma concentrations of total ADR after i.v. administration. Linkesch et al. (2001) speculated that Caelyx remains stable in the blood for at least 14 days. Secondly, there is passive targeting of LADR to the mononuclear phagocyte system such as the liver, lungs, lymph nodes and marrow, the tissues most susceptible to the dissemination of breast cancer. Thirdly, Caelyx provides a high accumulation of ADR in primary tumors (Koukourakis et al. 2000) and their distant metastases (Symon et al. 1999).

A serious problem in breast cancer therapy relates to the fact that the metastatic nodes outside the axilla cannot be resected in modified radical mastectomy, the surgical procedure applied most extensively. The solution is likely to be lymphatic chemotherapy, which provides the opportunity of delivering high concentrations of cytotoxic agents to lymph nodes over a prolonged period and thus should improve the outcomes, as shown in clinical studies on cancers of the digestive tract (Akamo et al. 1997, Hagiwara et al. 2000, Huang et al. 2002, Kin et al. 2002). Study of lymphatic targeting indicates that the administration of small-sized LADR by the s.c. route may provide enhanced efficacies on metastasized lymph nodes compared with by the i.v. route, supporting the potential of the approach in the treatment of local-regional nodes clinically involved with metastatic disease or, indeed, clinically uninvolved lymph nodes suspected of harboring micrometastatic disease. During recent studies on sentinel lymph node identification, there was clear evidence for the hypothesis that substances injected interstitially may imitate the patterns of lymphatic spread of breast cancer (Jansen et al. 2000, Eroglu et al. 2004).

Our studies also provided clear evidence for the conclusion that a more effective treatment of MBC could be expected by combining s.c. and i.v. administration. This combined treatment exhibits satisfactory chemotherapeutic activity in primary breast tumors and metastases in both local and distant tissues, as shown by the slowed growth of primary tumors and those in axillary nodes, by decreased PCNA mRNA expression and by extensive necrosis or
apoptosis of tumor cells in lungs and liver. Furthermore, because most of the LADR is localized in lymphatic tissues after s.c. injection and only a small fraction can pass through the lymphatics and reach the general circulation, it is reasonable to hypothesize that systemic toxicities may be substantially decreased compared with the i.v. route at the same dose. It is necessary, therefore, to gain more insight into the dose of total LADR that can be used in the combined therapy.

No evidence of local toxicity, such as allergy, ulceration or erosion of skin, was observed after s.c. injection and no animal died during the 1 week treatment, implying that the clinical application of LADR administered by the dual routes could be safe. Nonetheless, further investigation is still required to address the systemic toxicities that are probably induced by the treatment.

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