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# SHORT TIME EFFECTS OF RADIOTHERAPY ON LYMPHATIC VESSELS AND RESTORATIVE LYMPHATIC PATHWAYS: EXPERIMENTAL APPROACHES IN A MOUSE MODEL

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*ABSTRACT*

*Radiotherapy (RT) is an important component in the therapeutic approach to oncologic conditions. This study presents the investigative results on the impact of RT on lymphatic vessels and on the regenerative response of the lymphatic system in a mouse model. We first irradiated 3 groups of ten mice using brachytherapy in a single treatment of 20 Gy. We then performed morphological examination of the irradiated lymphatic vessels using an in vivo microscopic transillumination technique at 2, 4, and 6 weeks. Next we evaluated lymphatic flow using lymphoscintigraphy and in vivo microscopy at 6 to 11 weeks in: 10 additional mice following irradiation as above (IR), in 10 mice following incision of a lymphatic vessel (I), and in a non-treated control group of 10 mice (N). Intact lymphatic vessels were observed in all mice at 2, 4, and 8 weeks following the single dose of radiotherapy in the first group of mice and normal lymphatic flow was fully restored in the irradiated (IR) and incised (I) mice indicating that the reparative substitution lymphatic pathways are functioning normally. We found that following irradiation with one dose of 20 Gy, lymphatic vessels were not visibly damaged and also that lymphatic flow was consistently restored and substitutive lymphatic pathways formed.*

**Keywords:** Lymph, lymphatic vessels, lymphangiogenesis, radiotherapy, lymphoscintigraphy, lymphatic anastomosis, edema

In the aftermath of cancer treatment, radiotherapy (RT) is often mentioned as a secondary reason for the deterioration of lymphatic vessels. Irradiation of lymphatic vessels during the postoperative recovery process inhibits the development of these vessels and hence creates edema (1). To determine the impact of RT on the onset of edema, this study investigates the influence of RT on lymphatic vessels and lymphatic regenerative processes (lymphangiogenesis).

## *MATERIALS AND METHODS*

### *Lymphatic Vessel Investigation*

A lymphatic vascular mouse model was used to investigate the lymphatic vessels of the lateral superficial epigastric vein which connects the inguinal to the axillary lymph nodes (*Fig 1*). Studies utilized female white

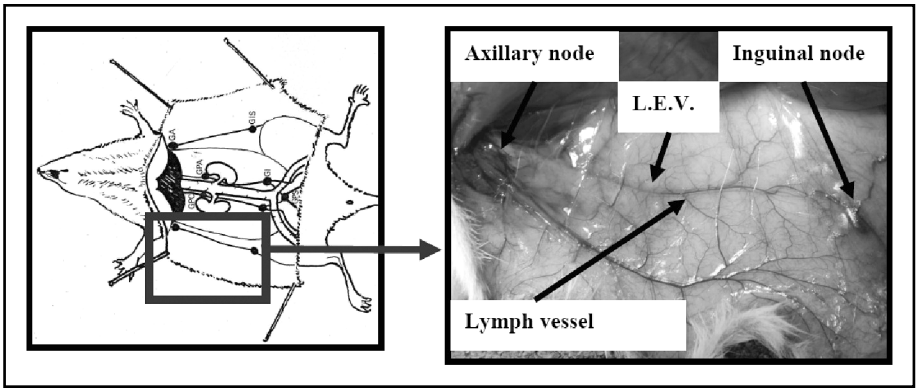


*Fig. 1. Anatomic diagram (left) and operative dissection (right) displaying the inguinal lymph node, lymphatic vessel (highlighted with Evans blue), axillary node, and lateral superficial epigastric vein (L.E.V.) structures used for the single dose experiments.*

mice, aged 6 to 8 weeks with average weights of 28 to 30g which were obtained from the Naval Medical Research Institute (Bethesda, Maryland, USA). Three groups of 10 mice were irradiated with a single dose of 20 Gy by brachytherapy.

A microscopic *in vivo* transillumination technique was used to investigate the subcutaneous microcirculation 2 weeks post-irradiation in the first group, and 4 and 8 weeks respectively in the second and third group. Anesthetized mice (urethane 25%) were opened and the skin of the right hemi-abdomen was shaved and then reflected back for visualization of the lymphatic pathways connecting the inguinal to the axillary node. Evans blue solution was either subcutaneously injected above the popliteal area before reflecting the skin or injected directly in the inguinal node once the abdomen was opened (2,3). The microcirculation was photographed for analysis (*Fig. 2*).

*Lymph Flow Evaluation*

A second set of 3 groups of ten anesthetized mice were prepared to evaluate lymph functional flow between the inguinal and the axillary node using two complementary investigative techniques to observe normal regenerating processes at 6 to 11 weeks. A non-treated control group of ten mice (N) was compared to a second group of 10 mice (I) who had their lymphatic vessel transversely incised and to a third group (IR) in which the lymphatic vessel had been incised and irradiated with one dose of 20 Gy by brachytherapy 15 days after the incision.

Lymphoscintigraphy was performed to evaluate the quantitative aspect of lymphatic function. Each anaesthetized mouse (cocktail of ketamine and medotomidine) was positioned on specially designed support. A volume of 0.2 ml of human serum albumin nanocolloid marked with metastable Technetium 99 (radioactive activity of 14 mCi per bottle of 1ml of HSA 99 Tc) was injected subcutaneously into the distal posterior part of the right thigh just above the popliteal area and the injection site was massaged for 5 minutes. A gamma camera was placed above the mouse and two dynamic acquisitions of scintillations of 120 seconds were collected with an interval of 2 minutes. Following a second massage of 5 minutes, a final series of dynamic acquisitions was collected for 120 seconds (*Fig. 3*).

Following collection of lymphoscintigraphic images, the subcutaneous microcirculation was examined by microscopic *in vivo* transillumination as above.

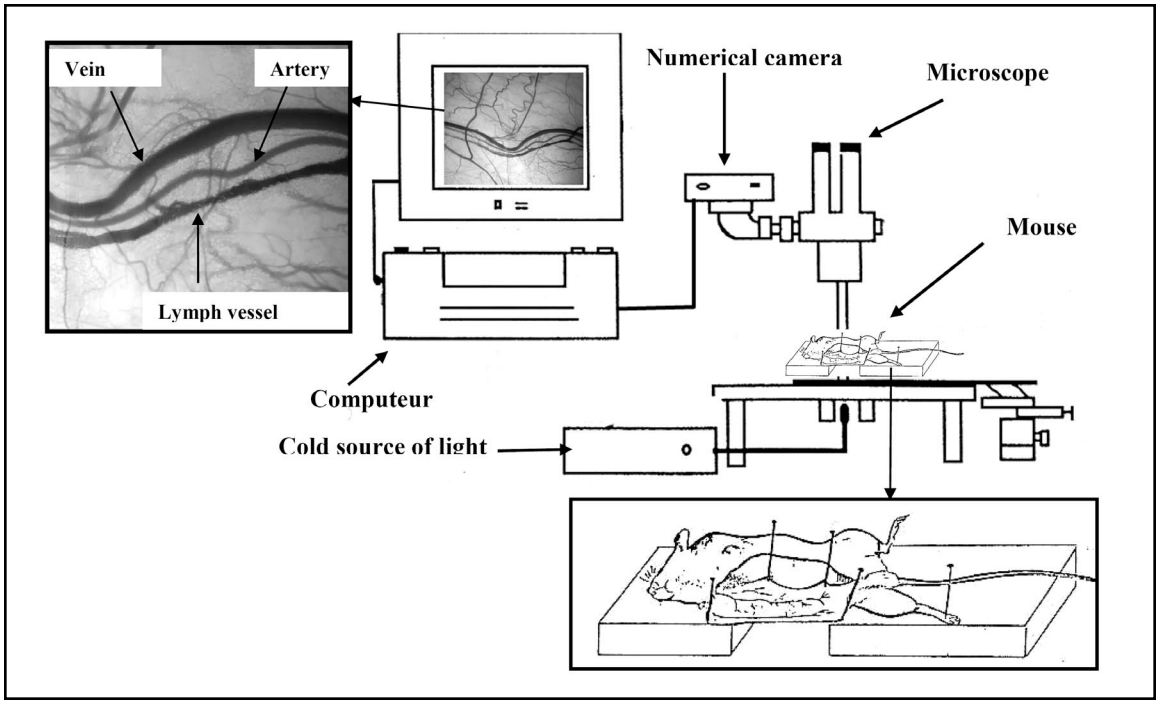


*Fig. 2. Microscopic* in vivo *transillumination technique and device used for visualization of subcutaneous abdominal vessels following Evans Blue injection.*

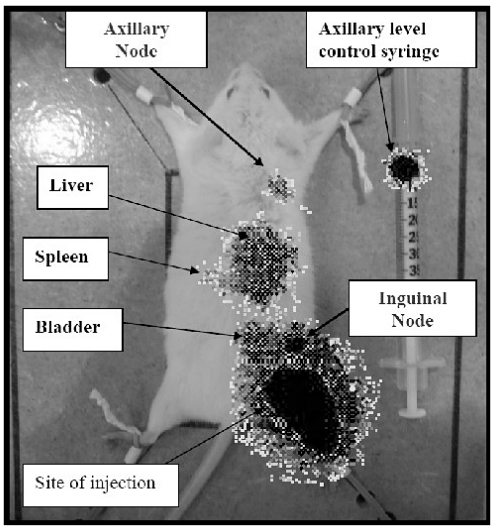


*Fig. 3. Lymphoscintigraphy results superimposed on a photograph of a mouse laying under the gamma camera after injection of tracer into the distal posterior part of the right thigh above the popliteal area. This mouse was in the IR group and displayed transport to the axillary node.*

## *RESULTS*

### *Lymphatic Vessel Investigation*

In all three groups external skin changes in the zone of irradiation were observed. In the first group, 2 mice died two weeks following the single irradiation session. Edema was seen in 4, significant redness in 7, and hair-growth inhibition in 2 out of the 8 surviving mice. In the second group, and 4 weeks post-irradiation, 2 mice had died. There was no evidence of edema or redness, but significant hair-growth inhibition was present in the 8 surviving mice. In the third group and 6 weeks post-irradiation, 8 out of the 10 mice evidenced an inhibition of hair growth, substantial scaling of their skin, but no edema. When the inner side of the skin was examined, edema was observed in 5 out of 8 mice in the first group and in 3 out of 8 in the second group. No edema was present

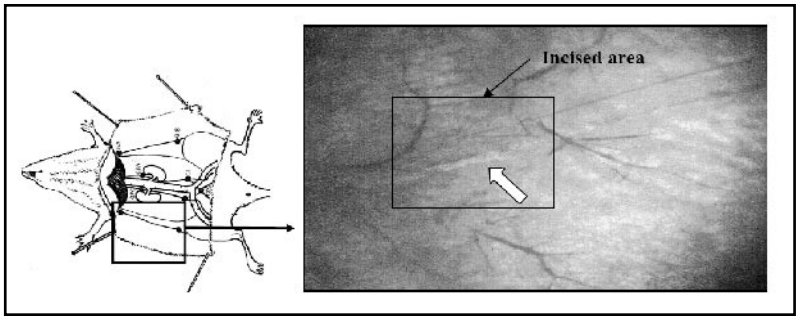


*Fig. 4. Microscopic view of a lymphatic vessel visualized by Evans blue in a mouse 8 weeks post irradiation. Vessel is visualized crossing the irradiated area. The image also highlights the area for incision used in the lymph flow experiments (I and IR groups).*

in the mice of the third group. Due to strong adhesions, difficulties were encountered in reflecting the skin in 4 out of 8 mice in both the first and second group. No adhesive problems were encountered in the 10 mice after an interval of 6 weeks post-irradiation. All mice in the three irradiated groups evidenced an intact lymphatic vessel and a normal passage of dye through that vessel in the irradiated area (*Fig. 4*).

*Lymph Flow Evaluation*

The interpretation of results concerning the lymphatic function is limited to 6 weeks after incision (Group I) and 4 weeks after irradiation (Group IR). Due to small numbers of surviving mice in each group, limited results were obtained as follows:

Using lymphoscintigraphy, the homolateral axillary lymph node was visualized in all surviving mice – 5 mice of the incised group (I) at 6 weeks observation and 6 mice of the joined incised/irradiated (IR) group 4 weeks after irradiation. The passage of functional lymphatic flow between the inguinal and the axillary node was re-established in all surviving mice. The lymphatic transfer capacity (LTC) of the radioactive tracer was measured in each mouse. This parameter of lymphatic function was obtained by relating the nuclear activity of the axillary node to the total nuclear activity of the mouse. The

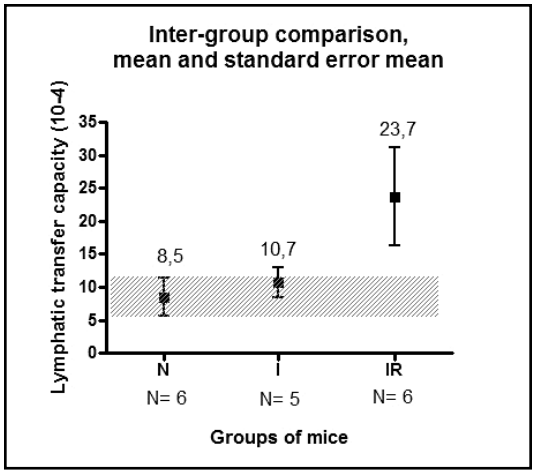


*Fig. 5. Lymphatic transfer capacity calculated from images obtained after the second massage 6 weeks post incision.*

results obtained in both the I and IR group 6 weeks following incision of the lymphatic vessel are summarized in Figure 5. While the LTC of the joined incised/irradiated mice (IR) was somewhat higher than the LTC in the control group (N) and in the incised mice (I), statistical analysis indicated no significant difference between the lymphatic transfer capacity of the "IR mice" and the "I mice" (Mann Whitney test, P value = 0.1775), and no significant difference between the lymphatic transfer capacity of the "IR mice" and "normal mice" (Mann Whitney test, P value = 0.1775).

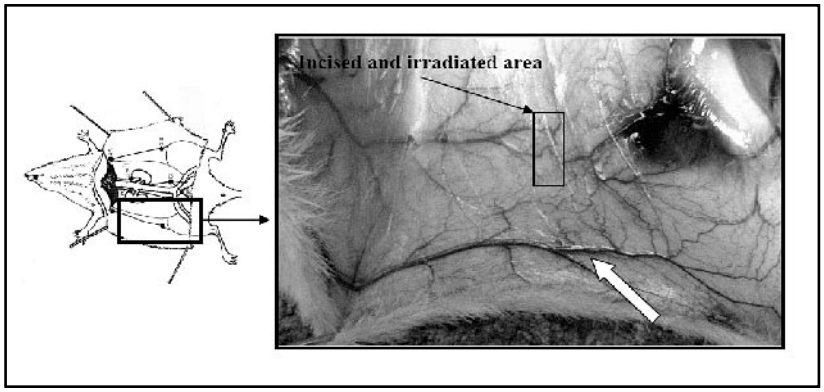


*Fig. 6. Visualization of a lympho-lymphatic anastomosis by direct dye injection into the inguinal node in an IR mouse (6 weeks post-incision and 4 weeks post-irradiation). Arrow highlights lymphatic vessels from the inguinal node joining the lateral lymphatic vessel.*

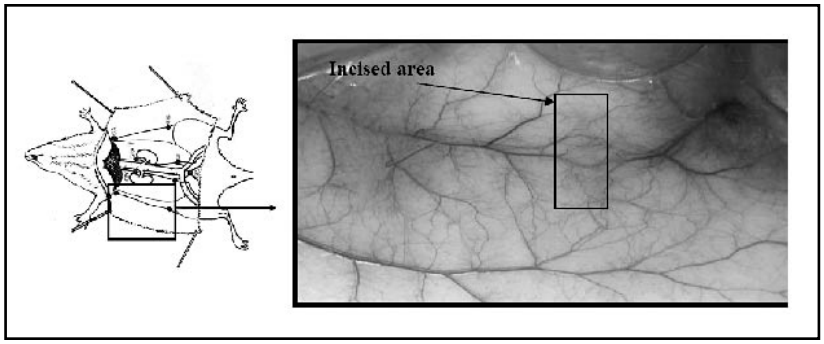


*Fig. 7. Neoformation of lymphatic network following indirect dye injection and visualization of the inguinal node in an I mouse (11 weeks post-incision) demonstrating a new network of lymphatic vessels connecting to the original lymphatic vessel.*

Microscopic *in vivo* transillumination examination of lymphatic pathways following visualization with Evans blue displayed lymph flow restoration between the inguinal and axillary lymph nodes. In all 10 mice – 5 out of 5 in both the I and the IR groups as one IR mouse died following lymphoscintigraphy – passage of the dye is seen between both lymph nodes following injection of Evans blue. This visualization is compatible with the lymphoscintigraphic results. Restoration of lymph flow between both nodes occurred by two mechanisms: A) In one "incised" mouse and in two "IR" mice, a lympho-lymphatic anastomosis (L) formed between the distal part of the interrupted vessel and another lymph vessel draining towards the axillary node (*Fig. 6*). Statistical analysis (Fisher test: P value = 1.000) did not indicate a significant difference between the rates of lymphatic anastomotic appearance between these mice groups. B) In all 5 "incised" I mice and in 3 out of 5 IR mice, the interrupted lymph vessel regenerated. Two mechanisms for lymphatic regeneration were encountered in the scarred area: 1) by a newly formed network of small lymphatic vessels (*Fig. 7*), or 2) by regeneration of the vessel in its initial form (*Fig. 8*). Statistical analysis (Fisher's exact test: P value = 0.4444) did not reveal a significant difference between the rates of regeneration of the incised vessels

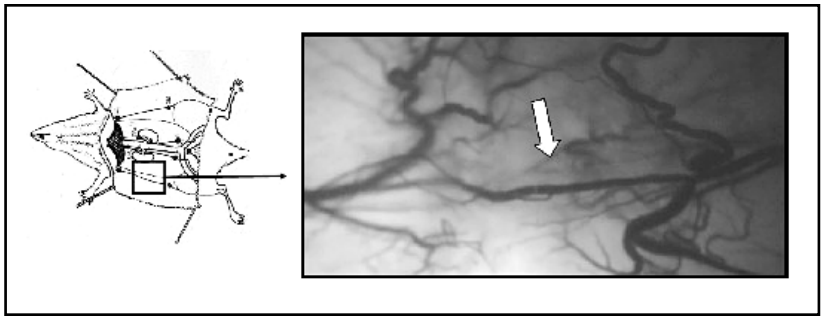

*Fig. 8. Microscopic view of a regenerated vessel in its initial form after direct injection of Evans Blue in the inguinal node in an IR mouse (6 weeks post-incision and 4 weeks post-irradiation).*

in both mice groups. It is interesting to note that a collateral or contralateral lymphatic pathway was never observed nor any lympho-venous anastomosis.

## *DISCUSSION*

The choice of this mouse model proved to be complex. The model allowed the lymphatic vessel connecting two lymph nodes to be easily visualized by direct or indirect injection of Evans blue dye. However, the fragility of these small animals presented a considerable disadvantage. The mice were very sensitive to changes in temperature and easily developed hypothermia leading to death during anaesthesia and in the post-interventional period. Because of this potential loss, future investigations should consider an increased number of mice as well as precautionary measures to reduce hypothermia.

Cutaneous incisions to generate lymphangiogenesis do not induce drastic aseptic conditions and are rather easy to perform. A healing process in surgically interrupted subcutaneous lymph vessels has been created frequently for studies on lymphatic regeneration, and the development of a new network of small lymphatic vessels occurs even in scar tissue after incisional procedures. Prior studies have shown that the regeneration of lymphatic collectors is seen until the end of the 2nd post-incisional week (4-6).

Brachytherapy is an appropriate technique of radiotherapy to irradiate a small cutaneous area. It offers the opportunity to deliver great doses of irradiation to a well delineated zone while the surrounding tissues are spared as much as possible. Studies on the effects of irradiation on lymph vessels date back to 1991 when Mortimer irradiated lymphatic vessels using one 18 Gy dose (7). Since then, irradiation techniques have evolved. In the current study, following calibration tests we were able to deliver a dose of 20 Gy accurately to the incision zone. Radiation results in our mouse model cannot be extrapolated to human beings. Although the irradiation methods are similar to treatments in human cancer, the intensity is different in that radiation is delivered in primarily fractioned doses to humans.

Lymphoscintigraphy is frequently used to evaluate lymphatic function qualitatively and quantitatively (8). Manual massage at the site of injection is performed in humans to obtain uniform tracer transit times between the lymph nodes. Indeed, the transit times decrease between the knee and the groin during indocyanine green (ICG) fluorescence lymphography when the injected area is massaged compared to the situation of rest without massage (9).

In this study, it would have been preferable to inject only a volume of 0.1 ml of radioactive tracer instead of 0.2 ml at the injecting site. A smaller dose could have

facilitated the localization of the inguinal node and increased the quality of correction of the measured nuclear activity in the inguinal node. Because of the high values, nuclear activity in the inguinal and axillary nodes could not be calculated and inter-related appropriately. Therefore, we decided to relate the ratio of nuclear activity at the axillary node to the overall nuclear activity of the mouse. To obtain exact figures in both lymph nodes following lymphoscintigraphy, immediate dissection of these structures, identified by Evans blue injection, is essential to measure their nuclear activity with a miniaturized gamma camera (3). However, full dissection of all lymphatic structures was not considered as *in vivo* visualization of the subcutaneous vessels by transillumination would not have been possible.

*In vivo* visualization of subcutaneous vessels by transillumination in the anaesthetized mouse is interesting to obtain quantitative dynamic data of the lymphatic microcirculation. If the animal is sacrificed, only the anatomical aspects could have been considered.

In the first part of this study, we found that our experimental protocol was efficient to analyze the potential destruction of the irradiated lymphatic vessel. Following the injection of Evans blue, visualization and identification of following structures were made possible:

- structures representing lymphatic regeneration following post-irradiation destruction of the primary lymphatic vessel: the presence of a network of small newly formed vessels, the presence of lympho-lymphatic anastomoses, and/or collateral lymph vessels,
- structures indicating an intact primary lymphatic vessel: the presence of a single lymphatic vessel, the absence of lympho-lymphatic anastomosis and collateral vessels, and absence of increased permeability as evidenced by escape of dye from the vessel.

The results in the second part are open to differences in interpretation as it is impossible to conclude that destruction by irradiation of the post-incisional lymphatic network takes place. Indeed, imaging evidence is lacking to show the evolution of the newly formed network immediately (1, 2, 3 days) following irradiation rendering it impossible to know whether this network remains intact or is destroyed. If a primary lymphatic network (R1) of small vessels is destroyed, a secondary (R2) could have appeared 4 and 9 weeks after irradiation. For imaging the immediate post-irradiation evolution of a lymphatic network, the mice would have to be sacrificed for dissection and visualization of the lymph vessels. This could be realized by injecting fluorescent indocyanine green (ICG) before and after irradiation once the incision is performed. The effects of radiotherapy on the evolution of the lymphatic network in scar tissue and on lymphatic regeneration could then be observed on photographs.

The results in the first part of this study indicate that no lymphatic damage is observed 2, 4 and 8 weeks after a one dose irradiation of 20 Gy delivered by brachytherapy. These results are compatible with earlier studies of Engeset (10) and of Sherman and O' Brien (11) who have shown the resistance to damage of lymphatic vessels following radiotherapy at a dose of 10 to 30 Gy delivered in one dose.

Many mice died during the second part of the study, only allowing conclusions from the results 4 weeks after irradiation. The authors attempted to answer the following questions and were able to obtain some insights into the answers;

*In the short term, does restoration of the lymphatic flow in an incised vessel depend on previous irradiation?* No. Restoration of lymphatic function was consistently observed following irradiation of a regenerating incised vessel. Transfer of lymph between the inguinal and axillary nodes was not impeded.

*In the short term, does irradiation*

*increase the appearance of lympho-lymphatic anastomosis or collateral pathways?* No. Statistically the number of substitute pathways did not increase after radiotherapy. Nevertheless, the number of anastomoses was higher than in the normal non-irradiated situation (0 in the normal mice). It is very crucial after irradiation in humans to sustain, within a short time after an intervention entailing ablation of lymph nodes, the opening and functioning of these substitute lymphatic pathways.

*In the short term, does there exist any difference in the lymphatic transfer capacity between the incised and irradiated (IR) mice, the incised only mice (I), and the normal mice (N)?* The lymphatic transfer capacity in the incised and irradiated mice (IR) was not increased significantly to the capacity in the control (N) and the only incised mice (I) possibly due to low numbers of animals (*Fig. 5*). After 4 weeks, the transfer capacity in I mice was the same as in the N mice. Indeed, after 4 weeks, lymphatic regeneration was restored and functional. It is not surprising that the lymphatic transfer capacity in IR mice seems to be higher than in N mice. Mortimer (7) observed a significant increase in lymphatic clearance in the cutaneous zones of pig skin 6 weeks post-irradiation compared to the lymphatic clearance in the same zones before irradiation. The results in our study were not statistically significant for an increase in the lymphatic transfer capacity. Not only was the number of mice too small but the increase of lymphatic transfer capacity in mice might not have reached its maximal value at 4 weeks post- irradiation compared to the 6 weeks in Mortimer's study.

In this study, the lymphatic transfer capacity (LTC) of the regenerated network (R1 or R2) was at least equal to the LTC of the lymphatic vessels before the intervention. Short-time results were obtained only after one irradiation dose of 20 Gy on intact lymphatic vessels and on lymphatic vessels following incision. After irradiation, it is possible that in the long term intrinsic and extrinsic stenosis of these lymphatic vessels may occur by fibrosis of the interstitial tissue as occurs in blood vessels (12,13). Analysis of markers of cutaneous fibrosis such as transforming growth factors (TGFs) and histological examination of lymphatic vessels could substantiate that stenosis is occurring.

## *CONCLUSION*

This mouse model demonstrates the short-term resistance of lymphatic vessels to irradiation of 20 Gy delivered in one dose by brachytherapy. The protocol does not allow for conclusions about short-term resistance in the newly formed lymphatic vessels. There is no statistical evidence that lymphatic function increases after irradiation. However, its presence is comparable to normal lymphatic function.

New substitute pathways (lympho-lymphatic anastomosis and lymphatic collaterals) were evident. It is tempting to suggest that these substitutive pathways might assume importance in therapeutic approaches in humans who have undergone surgery involving section and ablation of the lymphatic system followed by irradiation. Indeed, post-intervention physical treatments (Manual Lymphatic Drainage) may further promote these substitute lymphatic pathways.

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