Lymphology 32 (1999) 111-117

REFINEMENT OF A RODENT MODEL OF PERIPHERAL LYMPHEDEMA

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ABSTRACT

A reliable, inexpensive experimental counterpart of peripheral lymphedema has been notoriously difficult to reproduce thereby stifling basic and clinical research into this frustrating clinical condition. Accordingly, in 45 adult Wistar-Fuzzy rats, we attempted to produce sustained hindlimb lymphedema by either groin nodal/lymphatic microsurgical ablation (S) (guided by visual blue dye lymphography) or limited field-groin irradiation (R) alone (4500 rads) or combined S followed by R or R followed by S with an additional non-manipulated group serving as controls. Observations were made for 30-100 days thereafter. Hindlimb volumes were determined serially using the truncated cone formula based on multiple circumferential measurements at standardized intervals along the affected hindlimb and the findings compared with similar measurements in the contralateral non-manipulated hindlimb. In randomly selected rats from each group, lymphatic drainage was assessed by lymphangioscintigraphy (LAS), soft tissue swelling by magnetic resonance imaging (MRI), and edema fluid total protein content by refractometry. Whereas S or R alone produced only transient or mild hindlimb edema without associated morbidity or mortality, S-R or R-S induced moderate to severe sustained protein-rich hindlimb lymphedema associated with 9-13% early mortality and notable late local limb morbidity. Lymphatic obstruction was documented by sustained maintenance of increased hindlimb volume, subcutaneous fluid accumulation (MRI), and impaired lymphatic drainage (LAS). This reproducible rodent model of secondary lymphedema reliably simulates a stable clinical condition for a window of up to 100 days and should thereby facilitate standardized testing of therapeutic/preventive protocols and basic research into lymphatic dynamics in secondary lymphedema.

Secondary extremity lymphedema is a common long-term complication of successfully managed breast and pelvic cancer when regional lymphadenectomy and/or irradiation has been performed. Whereas accumulation of protein-rich interstitial edema is a direct consequence of the imbalance between net blood capillary filtrate (lymph formation) on the one hand, and reduced absorption into the compromised regional lymphatic apparatus on the other hand, the detailed and variable pathophysiologic, biochemical and molecular events within the microcirculation and interstitium during lymph stasis remain incompletely understood (1). Moreover, although a variety of physical and operative measures are reported to reduce or minimize the volume of lymphedema, detailed information on how these treatment protocols work and their

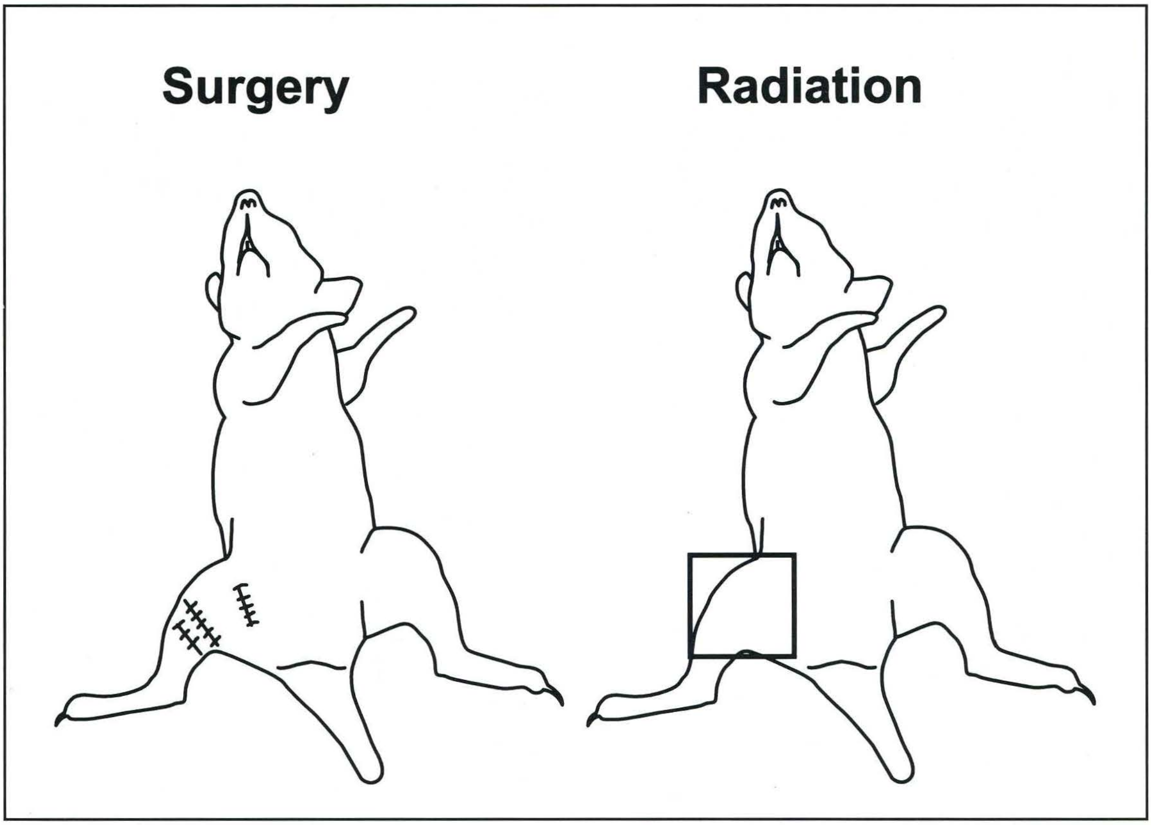

Fig. 1. Schematic representation of procedure for microsurgical ablation of right groin lymphatics and nodes (Surgery, left) and single dose 4500 rad medium field right groin irradiation (Radiation, right). See text for further methodologic details.

relative efficacy is limited. Accordingly, progress in developing and testing preventive and therapeutic approaches to this life-long disabling condition has been hampered.

A reliable and inexpensive method of reproducing lymphedema in a small animal as an experimental counterpart of the human condition would go a long way toward filling in gaps in fundamental knowledge and would also allow controlled testing of preventive and treatment approaches in this disorder. Previous efforts have been plagued by high cost for acquisition and long-term maintenance of large animals, lack of reproducibility, requirement of multiple operative manipulations to interrupt regenerating lymphatic pathways, protracted time before lymphedema becomes sustained and irreversible, and spontaneous resolution of swelling by vigorous formation of new lymphatic channels (lymphangiogenesis) (2-6). In 1990, a model of chronic lymphedema in Sprague-Dawley rats was described by Kanter et al consisting of unilateral regional groin irradiation followed by extirpative lymphatic surgery (the reverse of the usual clinical sequence) (7). Whereas the protocol was partially successful, followup outcomes and further experiments using this technique have not been reported. Accordingly, after reevaluating this experimental design in a pilot group of Sprague-Dawley rats, we applied a refined protocol to Wistar-*Fuzzy* (W-FU) rats, whose hypotrichosis facilitated limb manipulation, examination, and measurement. After unilateral microsurgical ablation of regional groin lymphatics and lymph nodes and limited field single dose irradiation alone or in combination with surgical excision, lymphatic blockage was assessed by lymphangioscintigraphy, soft tissue swelling by magnetic resonance imaging, serial changes in hindlimb volume by circumferential hindlimb measurements, and protein composition of edema fluid by refractometry. The findings were compared with those in the non-manipulated contralateral hindlimb.

MATERIALS AND METHODS

According to a protocol approved by The University of Arizona Institutional Animal Care and Use Committee, 45 adult Wistar-*Fuzzy* (W-FU) rats were divided into 4 experimental groups: Surgery (S), Radiation (R), Surgery followed by Radiation (S+R) and Radiation followed by Surgery (R+S) and an additional non-manipulated control group.

Operative preparation consisted of unilateral microsurgical ablation of regional groin lymphatics and lymph nodes using a dissecting microscope for magnification (Weck ~15x) *(Fig.1, left)*. Under ketamine-acepromazine anesthesia, a vertical incision was made in the mid-groin, after intradermal 1% Evans blue dye injection into the dorsum of the right foot. Femoral lymphatics and adjacent groin lymph nodes were readily identified by blue staining *(Fig. 2, upper left)*. These lymphatics and nodes were excised and the femoral blood vessels skeletonized. In addition, all visible and non-visible surface lymphatic pathways were disrupted by a circumferential skin incision, and the skin edges were sutured to the fascial muscle layer leaving a 5-8mm integumentary gap. Furan antibiotic spray was lightly applied to the slightly open wound.

Irradiation consisted of 4500 rads (4.5Gy) delivered in a single dose to the right groin (2.5x2.5cm field) of anesthetized rats using a Cobalt-60 gamma machine (Atomic Energy of Canada Limited, Theraton 80 model) *(Fig. 1, right)*.

The combined surgery-radiation (S+R) and radiation-surgery (R+S) sequences were separated by an interval of 3 days.

Rats were observed for at least 30 days and up to 100 or more days. Body weight was measured at least weekly and hindlimb volumes estimated serially using a modified truncated cone formula (8) based on 2 circumferences taken with a soft thin measuring tape at standardized intervals [2 cm below the "heel" (C1) and 1 cm above the heel (C2)] along the experimental and contralateral (control) limb.

In three additional rats undergoing the combined treatment sequence, the distribution of soft tissue swelling and fluid accumulation was further documented by magnetic resonance imaging (MRI) in coronal and sagittal sections of both hindlimbs using a 4.7 Tesla specially adapted small animal experimental magnet (Brükker). In nine additional rats undergoing the combined treatment, lymphatic drainage was assessed by bilateral hindlimb lymphangioscintigraphy (LAS) using Tc-99m human serum albumin (100 microcuries or 3.6 MBq in 0.1 ml) as the radiotracer injected intradermally into the dorsum of the hindpaw (9). Serial whole-body images were captured on a parallel hole collimator at minute intervals by a Toshiba Model 901 gamma scintillation camera and displayed as 15 and 30 minutes radioactive count accumulations. Edema fluid was also obtained from the swollen hindlimb in 29 combined treatment rats by needle aspiration and the total protein content (American Optical TS refractometer) measured and compared with levels in blood plasma.

RESULTS

The operative procedure alone (S) or irradiation alone (R) were well-tolerated and unassociated with mortality or early or long-term morbidity after the experimental manipulation. When surgery and irradiation were combined in either sequence, however, mortality was substantial (9-13%) and late

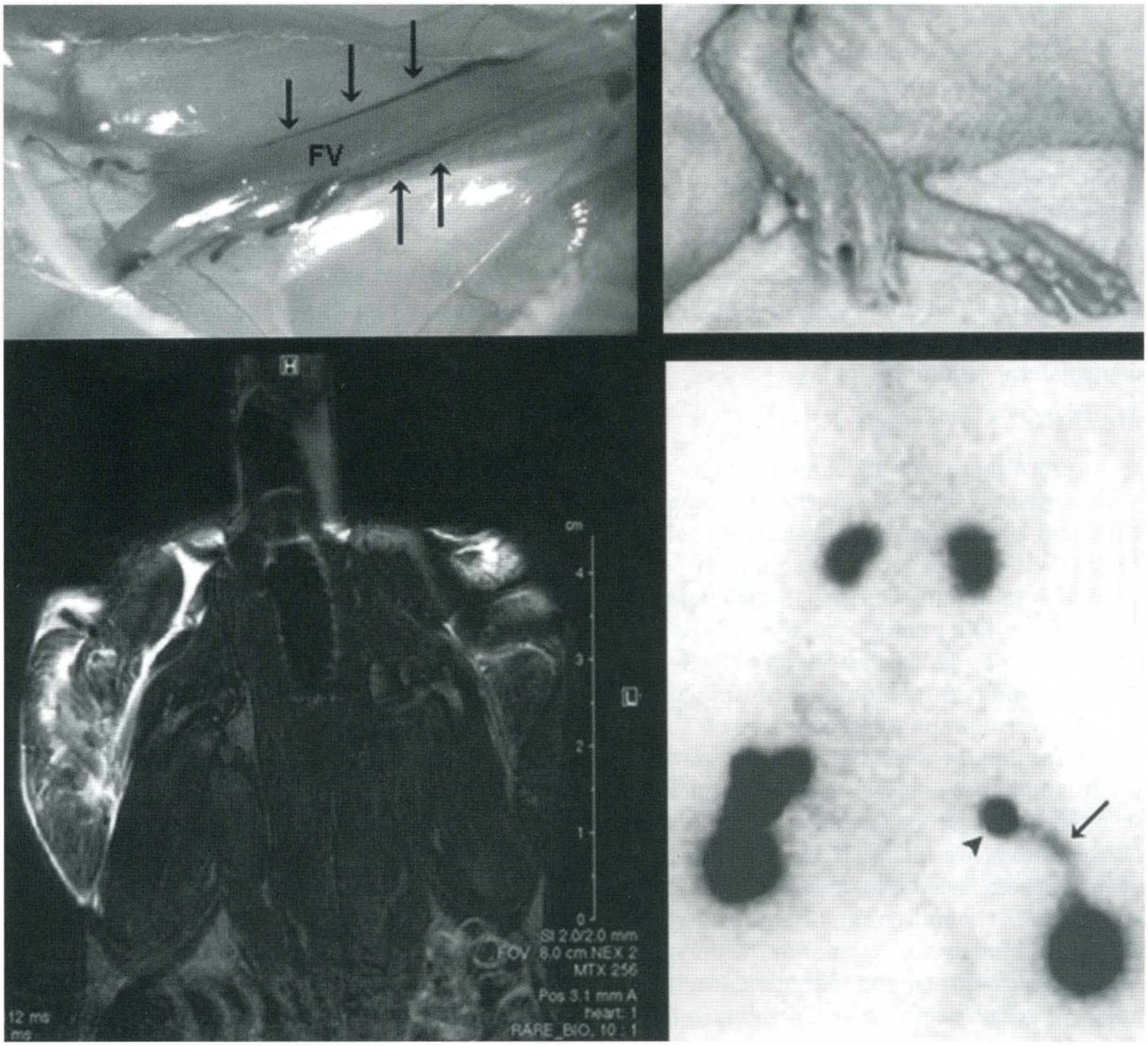

Fig. 2. Upper left, photograph of the initial step in the operative procedure (Surgery), where blue-stained femoral lymphatics (arrows) parallel the femoral vein (FV) and nodes are identified in the right groin and excised along with the regional lymph nodes. Subsequently, a 5-8 mm integumentary gap is created. Upper right, persistence of chronic right hindlimb lymphedema in a rat four months after undergoing combined surgery and groin irradiation. Lower left, T_2-weighted MRI (coronal section) through the hindlimbs (tail at top). The right limb (left side) was treated by combined groin surgery and radiation and shows typical findings of severe lymphedema 40 days later with increased fluid (white density) in the subcutaneous tissue and along fascial planes but sparing the skeletal muscle. The contralateral untreated left limb (right side) contains no detectable free fluid except for a small localized area due to anesthetic injection. Lower right, bilateral hindlimb lymphangioscintigram of rat with lymphedema of the right hindlimb one month after combined surgery and irradiation displaying hold-up of radiotracer in the right hindpaw (left side) with club-shaped dermal diffusion without visualization of lymphatic collectors or regional lymph nodes. In the normal left hindlimb (right side), tracer has migrated from the injection site into lymphatic trunks (arrow) with visualization of regional lymph nodes (arrowhead). The kidneys are also highlighted from tracer that has entered the central circulation.

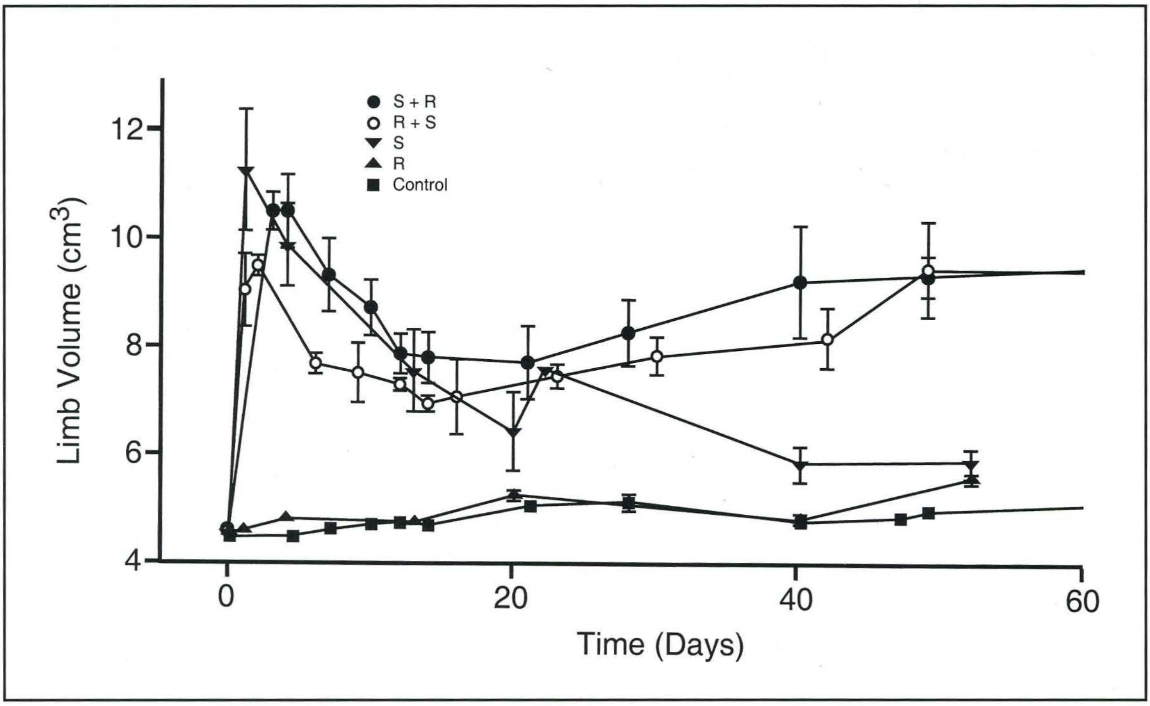

Fig. 3. Time course of limb swelling after surgery (S) and/or radiation (R). Note the sustained increase in limb volume only in S+R and R+S groups (mean±SEM).

morbidity (after 45-60 days) consisting primarily of limited or occasionally more extensive skin breakdown, soft tissue fibrosis, and bone necrosis in the lymphedematous limb was common. Body weight declined initially but increased thereafter in a linear fashion.

Edema of the experimental limb was readily visible promptly after lymphatic excision although swelling gradually subsided over the ensuing weeks when irradiation was not added. On the other hand, when irradiation was added either before or after lymphatic excision, the chronic swelling was not only greater, but even after an initial decline, was subsequently sustained and stable over the entire period of observation *(Fig. 2, upper right)*. Radiation treatment alone was not associated with overt edema.

Limb circumference measurements and calculated limb volumes [which were previously established to correlate closely (r=.92) with water displacement determinations] confirmed in quantitative terms the visual impressions. During the first week, calculated limb volumes increased sharply for the three groups that included lymphatic excision but only minimally or not at all for the radiation only group *(Fig. 3 and Table 1)*. On the other hand, the addition of irradiation to surgery shortly before or shortly afterward greatly increased in a sustained fashion the volume of hindlimb edema.

Magnetic resonance imaging *(Fig. 2, lower left)* showed a characteristic lymphedema distribution pattern of increased fluid in the subcutaneous tissue and fascial planes but not in the muscle compartment (subfascial) of the swollen hindlimb.

Lymphangioscintigraphy *(Fig. 2, lower right)* documented the typical club-shaped appearance of lymphatic obstruction with dermal tracer diffusion and absence of truncal and nodal visualization in the

TABLE 1
Hindlimb Volume (% increase* mean±SEM)

Group	n	7-11 days	17-21 days	> 30 days
R only	5	5.5±3.0	7.5±.2	-0.6±1.3
S only	5	54.4±18.0	35.1±13.3	11.5±3.2
R then S	10	84.5±11.1	54.9±6.9	54.9±6.9
S then R	15	101.3±9.3	59.4±11.8	55.9±8.0

*Control hindlimb compared with contralateral unmanipulated hindlimb.

lymphedematous hindlimb in contrast to the intact lymphatic trunks and regional nodes in the contralateral control limb.

Hindlimb lymphedema fluid was high in total protein content (3.49±.02 g/dl, mean±SEM) compared to plasma (5.58 ± .01 g/dl), consistent with the composition of lymphedema fluid.

DISCUSSION

Chronic lymphedema was first reproduced experimentally by Drinker et al (2) using intralymphatic injection of silica parti-cles, and later Danese, Olszewski, Clodius and their colleagues (3-5) demon-strated that radical lymphadenectomy with skeletonization of the femoral vessels led to transient then latent (subclinical) and eventually intractable peripheral lymphe-dema. These studies, however, were done in large animals, were difficult to carry out and would be prohibitively expensive by current standards. In searching for a simpler and cheaper experimental counterpart of the human disorder and one that was more rapid than the delayed rodent model proposed by Wang and Zhong (10), we opted to refine and further study a method first described by Kanter et al (7) in Sprague-Dawley rats using radical regional lymphatic nodal excision in conjunction with regional irradiation, modalities commonly used to treat human cancer and commonly complicated by later development of peripheral lymphedema. Using near hairless rats (Wistar-Fuzzy), we examined in various combinations the success rate of lymphatic ablation (facilitated by blue dye lymphography) and limited field regional irradiation (4500 rads). The findings substantiated that lymphatic regrowth potential (lymphangiogenesis) was considerable such that radical excision or nodal/lymphatic irradiation alone produced only transient hindlimb lymphedema. Only the combination protocol—excision promptly followed by irradiation or irradiation promptly followed by excision—was almost uniformly successful in rapidly producing moderately severe and sustained unilateral hindlimb lymphedema albeit accompanied by significant early mortality and delayed limb complications. Despite these drawbacks, the use of a small animal (namely the rat) was still economical when compared with large animals as many more rats could be prepared and studied at low cost. We were also able to document the structural and physiologic derangements of lymph transport in these lymphedematous hindlimbs by limb volume measurements (truncated cone method using-circumferential measurements), isotope lymphography (dermal backflow) and magnetic resonance imaging (skin and subcutaneous edema with relative muscle sparing). The success of this experimental counterpart in simulating the clinical condition as it often occurs in secondary lymphedema after operation and radiotherapy for cancer now makes it possible to generate a "colony" of rats for dissecting pathomechanisms and quantitatively assessing diagnosis, current and new approaches to therapy and prevention of lymphedema.

ACKNOWLEDGMENTS

Supported in part by a grant from Beiersdorf-Jobst, Inc. (CLW) and an NIH Minority Supplement to CA 73095 (LL). Presented in part at the Western and American Federation of Medical Research meetings in Carmel, CA, January 30, 1999, and Washington, DC, April 18, 1999.

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