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LYMPHSPIRATION

INVESTIGATION OF THE MECHANISM OF LYMPHOCYTE INJECTION THERAPY IN TREATMENT OF LYMPHEDEMA WITH SPECIAL EMPHASIS ON THE CELL ADHESION MOLECULE (L-SELECTIN)

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ABSTRACT

We previously employed intraarterial lymphocyte injection therapy in conjunction with standard non-operative treatment of peripheral lymphedema of various etiologies. In this study, we further evaluated the clinical outcome of this therapy in 46 patients with unilateral lymphedema of the extremities. The results showed combined therapy (lymphocyte injection with compression) was effective in 74% (34 of 46 patients) with dramatic reduction in lymphedema in 37% (17 of 46 patients). In the most recent 5 patients treated, we examined the expression of cell adhesion molecule of the lymphocytes (L-selectin) before, during and after lymphocyte injection therapy to study the putative pathomechanism of this treatment method. The expression of L-selectin, a lymphocyte-specific adhesion molecule, increased in the autologous lymphocytes obtained by a blood cell separator and in the lymphocytes from the peripheral blood after injection. Moreover, the lymphocyte fraction, which was positive for L-selectin and negative for CD3, a T-cell marker, decreased after lymphocyte injection. We postulate that the lymphocytes of L-selectin (+) and CD (-) remain in the affected swollen limb and play a role in an ill-defined immunologic responsiveness that potentiates reduction in edema.

Earlier we reported a dramatic effect of intraarterial injection of a suspension of autologous lymphocytes to potentiate reduction in swelling of lymphedematous limbs (1,2). Although some patients showed a rapid response to lymphocyte injection, the effectiveness was short-lived (2). Accordingly, other non-operative treatments such as massage of the lymphedematous limb, use of bandage-wrapping, and medication such as benzopyrone were also employed in conjunction with lymphocyte injection (3,4). In this paper, we describe the results of our treatment protocol which includes intra-arterial lymphocyte injection therapy in patients with unilateral limb lymphedema. To investigate the apparent efficacy of lymphocyte injection, we examined the expression of cell adhesion molecule of the lymphocytes in 5 patients with primary or secondary lymphedema of the legs. We had previously observed that intraarterial injected lymphocytes, remained for a prolonged period in the affected limb (3). We hypothesized that the efficacy of lymphocyte

TABLE 1
Results of Compression Therapy Combined with Intraarterial Lymphocyte Injection for Treatment of Unilateral Limb Lymphedema

ERR	Upper Extremities	Lower Extremities
<30%	4	8 (4)
30-60%	4 (2)	13 (5)
60%<	5	12 (4)
TOTAL	13 (2)	33 (13)

( ): primary lymphedema; ERR (edema reduction ratio)=A-D/Ax100 (%) where A=difference between circumferences of affected and healthy limbs on admission (cm) and D=difference between circumferences of affected and healthy limbs on discharge (cm).

therapy in ameliorating lymphedema derived from augmentation of the expression of a cell adhesion molecule of the lymphocytes, and, accordingly, in the 5 most recent patients with leg lymphedema, the expression of L-selectin, a lymphocyte specific adhesion molecule and of CD3, a T-cell marker, on the membrane surface of lymphocytes was measured before, during, and after intra-arterial lymphocyte injection.

CLINICAL FINDINGS

We reviewed 46 patients with unilateral lymphedema from various causes who had undergone intraarterial lymphocyte injection combined with other non-operative treatment *(Table 1)*. Edema reduction was quantified by examining the ratio of the reduced difference between the circumference of the affected limb and that of the contralateral non-edematous limb on discharge to the difference between the circumferences of both limbs on admission (edema reduction ratio or ERR). ERR was calculated based on the most swollen site (i.e., the site with the largest difference of circumference between the affected and unaffected limb) among 4 loci in both arms and legs (upper arm, forearm, wrist, hand; thigh, calf, ankle, foot). Each subject gave informed consent to participate in the study and the protocol was approved by the Ethics Committee of the University of Tokushima.

Technique for Intraarterial Lymphocyte Injection

After venous blood was obtained from an antecubital vein, autologous lymphocytes were isolated in each patient using a blood cell separator (Baxter CS3000). Promptly after separation, each patient was given a bolus injection (suspension 100 mL) of lymphocytes ($2x10^9$ cells) into a proximal artery of the affected limb. This treatment was repeated at least 4 times every 1 or 2 weeks. Other treatment of arm lymphedema included massage of the affected limb, use of bandages and oral benzopyrone.

L-Selectin Expression

In 5 patients *(Table 2)* with unilateral lymphedema of the leg, we examined lymphocyte expression of L-selectin before,

TABLE 2
Demographics of 5 Patients Undergoing Lymphocyte Measurement of Cell Adhesion Molecule (L-Selectin) After Intraarteral Lymphocyte Injection

Patient	Age	Gender	Affected Limb	Classification of Edema	Lymphocyte Injection
1	60	F	LL	2°	5x
2	64	F	RL	2°	6x
3	22	F	LL	1°	4x
4	56	F	RL	2°	4x
5	41	M	LL	2°	8x

LL=left lower; RL=right lower; 1°=Primary; 2°=Secondary

during and after intraarterial lymphocyte injection. Five ml of venous blood was drawn from the patient's femoral vein of the affected limb 1 hour before blood cell separation (control), just before intraarterial injection and 5 minutes after injection. A volume of 5 mL of cell suspension was also drawn from the mother lymphocyte suspension obtained by the blood cell separator (cell separated autologous lymphocytes). The blood and cell suspensions were then gently applied onto Lympholite H (Wako Pure Chemical Industries, Ltd., Osaka, Japan), a lymphocyte separation medium and centrifuged for 30 minutes at 400 g. The lymphocyte-rich fraction collected was washed two times with PBS solution, supplemented with 0.2% sodium azide and 2% fetal calf serum and resuspended and centrifuged for 5 minutes at 200 g.

Flow Cytometric Analysis

About $3x10^6$ lymphocytes from each patient resuspended with 50 µl of PBS were stained in an ice bath with shaded light for 30 minutes with FITC-conjugated anti-L-selectin monoclonal antibody (Seikagaku Co., Tokyo, Japan), plus RD-1-conjugated anti-CD3 monoclonal antibody (Coulter, Tokyo, Japan) at a concentration recommended by the manufacturer. These cells were subject to analysis by flow cytometry, FACscan (Becton Dickinson, Mountain View, CA, USA). Before analysis, the lymphocyte population was gated by light scatter signals to exclude dead and nonlymphoid cells.

Statistics

Values shown in *Figs. 1 and 2* represent mean ± S.D. Comparison of values was done by analysis of variance with post-hoc tests. A value showing $p<0.01$ vs. the control value was considered as statistically significant.

RESULTS

Thirty-four patients (74%) showed limb edema reduction of >30% and 17 (37%) showed >60% reduction in edema. No patient had worsening of edema or symptoms *(Table 1)*. The overall improvement persisted from several weeks to >5 years and no notable complications were encountered.

Table 2 shows the characteristics of the most recent 5 patients with unilateral leg edema who were examined the expression of L-selectin of lymphocytes before and after intraarterial injection. These values were compared with that of lymphocytes obtained by blood cell separation. As shown in *Fig. 1*,

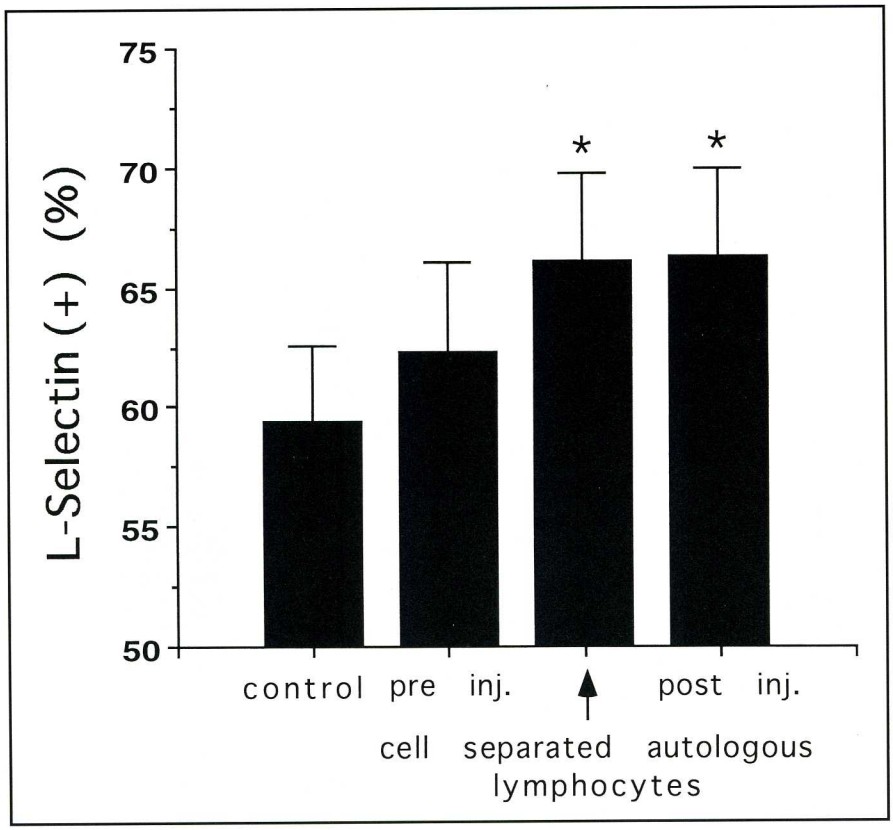

*Fig. 1. Changes in the expression of L-selectin on the lymphocytes before, during and after intraarterial (femoral) lymphocyte injection (control, pre-inj. and post-inj.). Lymphocytes were obtained from femoral unilateral venous blood 1 hour before blood cell separation, immediately before lymphocyte injection and 5 min after injection of cell separated autologous lymphocytes (lymphocytes derived from lymphocyte suspension by blood cell separation). *p<0.01 vs. the control value.*

the expression of L-selectin increased in the lymphocytes collected by the blood cell separator and also in the cells from the patient's venous blood after intraarterial lymphocyte injection.

To further characterize the L-selectin positive lymphocytes, we examined the expression of lymphocyte CD3 among the lymphocytes positive for L-selectin. As shown in *Fig. 2*, the fraction of lymphocytes were negative for CD3 among the lymphocytes positive for L-selectin, increased by blood cell separation, but decreased after intraarterial lymphocyte injection.

DISCUSSION

Intraarterial injection of isolated autogenous lymphocytes into a lymphedematous limb promotes short-lived but rapid diminution in interstitial edema (1,2). When this technique is combined with compression therapy such as massage manipulation and bandage wrapping, a prolonged benefit in lymphedema reduction is observed (3,4). Previously, after intraarterial lymphocyte injection, we observed a "novel" protein spot on a two-dimensional electrophoretic gel of the lymphedematous fluid (5). An *in vitro* study using cultured lymphocytes also demonstrated a similar protein spot on the gel of the edematous fluid. These observations suggested that the injected

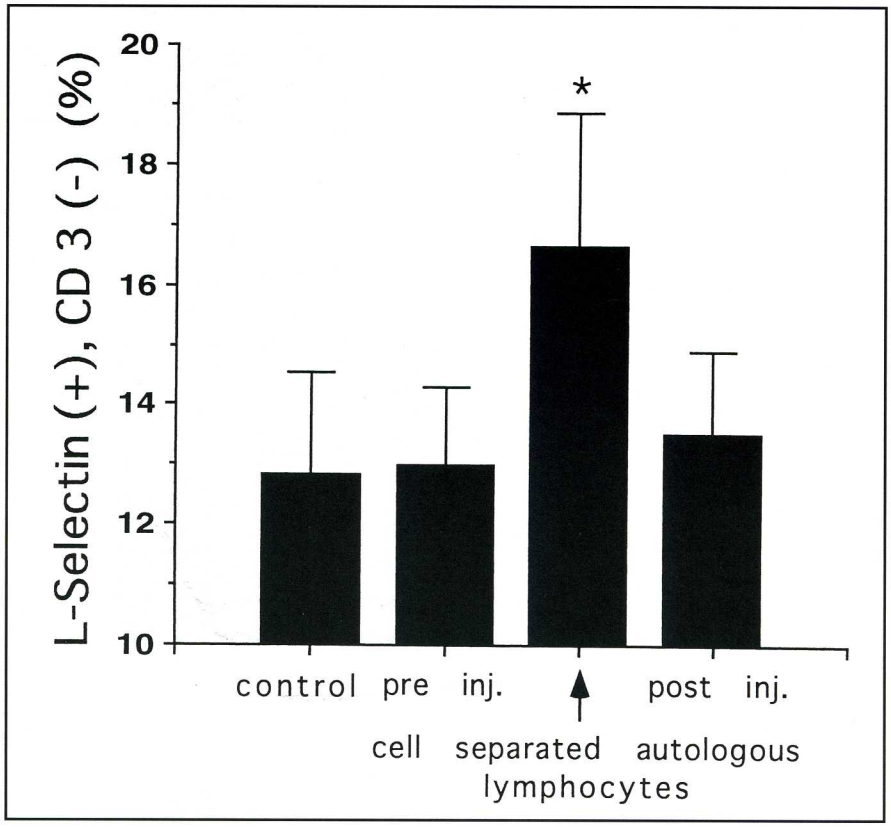

*Fig. 2. Changes in the fraction of lymphocytes positive for L-selectin and CD3 (-) before and after intraarterial lymphocyte injection (control, pre-inj. and post-inj.). Lymphocytes were obtained from femoral venous blood 1 hour before blood cell separation, immediately before the lymphocyte injection and 5 min after the injection of cell separated autologous lymphocytes (lymphocytes derived from lymphocyte suspension by blood cell separation). *p<0.01 vs. the control value.*

lymphocytes stimulated proteolytic modification of protein components of the lymphedematous fluid. Using radio-labeled autologous lymphocytes (3), we also found that some injected lymphocytes remained in the lymphedematous limb for at least 24 hours. Taken together, these findings suggested these residual lymphocytes in the lymphedematous limb interacted within the interstitium through the expression of adhesion molecule(s) which in turn promoted adherence to the vascular endothelium.

As shown in *Fig. 1*, the expression of L-selectin, a lymphocyte-specific adhesion molecule, increased in lymphocytes collected by a blood cell separator, and also in the lymphocytes obtained from unilateral femoral venous blood after femoral intraarterial injection. The lymphedematous leg findings may be the end result of shear stress to the lymphocytes induced by mechanical blood cell separation as shown by similar upregulation of L-selectin neutrophils to human umbilical vein endothelial cells (HUVEC) *in vitro* after shear stress (6). Neutrophil adhesion to endothelium *in vivo* is also regulated by L-selectin (8) including after shear stress (7) and in the bloodstream in L-selectin is also expressed on neutrophils (8). On the other hand, the increased expression in patients of L-selectin in the lymphocytes from femoral venous blood after intraarterial

injection is unclear, although it may reflect a local immunological reaction. In any event, the findings suggest that the intraarterially injected lymphocytes adhered to the blood vascular endothelium with upregulation of the L-selectin adhesion molecule. As shown by *Fig. 2*, among the L-selectin positive lymphocytes, the CD3 (-) cells likely represent the portion of lymphocytes adherent because the number of lymphocytes was reduced to near control level after injection. These lymphocytes probably extravasated into the interstitium (9). Other observations show that cutaneous lymphocyte-associated antigen is responsible for lymphocyte migration to the skin and subcutaneous tissue in response to inflammation or allergy (10). Whereas adhesion molecules other than L-selectin may be involved in the phenomenon of lymphocyte migration, the precise mechanism of the role that injected lymphocytes play is still speculative. After intraarterial injection of lymphocytes, there may be a significant interaction between the putative upgrading of adhesion molecules and the protein components of the lymphedematous fluid.

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