考察

筆者が最初に乳房縮小術の経験を重ねたのは，1991年頃の米国留学時代であった．当時の主流は，inferior pedicle & classic inverted T scar であった．Ribeiro，Courtiss & Goldwyn，Georgiade らの術式[1)~3)]に準じた縮小術の研鑽を積み，帰国後には巨大下垂乳房症例だけではなく，乳房再建症例の健側下垂乳房や乳腺腫瘍切除後の即時再建例に，その術式を応用してきた[4)5)]．その後，中程度～軽度の下垂乳房症例に対しては，central pedicle & short inverted T scar を用いた縮小術も施行してきた[6)]．

しかし，乳房下溝近隣に長い水平方向の瘢痕が残る inverted T scar に対しては，以前より批判があり，Marchac，Lassus，Peixoto らは，術後瘢痕をできるだけ小さくする試みとして，short inframammary scar の術式を報告してきた[7)~9)]．近年，欧米や南米から，vertical mammaplasty，vertical scar reduction mammaplasty として多くの報告[10)~14)]が見られるようになり，我々の施設でも，2000年以降は，主として superior pedicle & vertical scar による乳房縮小術を行ってきた．

本術式の注意点として，プリーツ状になる巾着縫合部の創治癒に時間を要することが挙げられる．創治癒が遷延した症例では術後瘢痕に対する保存的あるいは外科的処置が必要となるが，瘢痕部は乳房下溝に位置し，ほとんど目立たない．多くの患者が，長い水平方向の瘢痕が残る classic inverted T scar より，瘢痕が短い vertical scar を希望する．

最後に，非常に大きな乳房下垂の症例では，乳頭乳輪の血行を保持する pedicle 部分の volume により，縮小できるサイズに制限が生じる．患者が希望する十分な縮小を得るためには，pedicle 部分に相当する乳腺組織を含めた切除が必要となり，このような症例では乳頭乳輪を free composite graft として移動する術式が適応となる．

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Efficacy of Polidocanol Sclerotherapy for Capillary Malformation with Masked Venous Malformation

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Capillary malformations (CMs) generally present in childhood or young adulthood as a bluish or red discoloration of the skin. Pulsed dye laser (PDL) is the traditional treatment for CMs, but some lesions may be difficult to obliterate when they extend deeply.

Previous studies have proposed the effectiveness of the long PDL and the long-pulse 1,064-nm neodymium-doped yttrium aluminium garnet (Nd:YAG) laser. The 595-nm PDL is effective for the treatment of leg telangiectasia, spider veins, and vascular lesions that are less than 1.5 mm in diameter.[1,2] The long-pulse 1,064-nm Nd:YAG laser has been used successfully to treat deeper, larger diameter vessels such as leg veins,[3] but the effectiveness of long PDL is uncertain, and one study has shown that only three of 22 resistant capillary malformations improved with the long PDL in one treatment session.[4]

Furthermore, percutaneous sclerotherapy has not been a candidate among the treatment modalities for CMs. It is usually used in low-flow malformations such as venous malformations and macrocystic lymphatic malformations. Lesions with large vascular pools are ideally suitable for sclerotherapy.[5] We performed percutaneous sclerotherapy in a patient diagnosed with CM compounded with masked venous malformation using magnetic resonance imaging (MRI). The diagnostic procedures, therapeutic possibilities, and complications are reviewed.

Case Report

A 31-year-old woman presented with a CM involving the right cheek (Figure 1A). She had received more than 10 treatment sessions with PDL in another clinic, but had had a poor response in terms of the lesion's color. She was eventually referred to our clinic. No thrills or bruits were detected in her right cheek on physical examination.

The lesion was studied using MRI and duplex ultrasound to appreciate the true anatomic extent and components of the lesion. T2-weighted, fat-suppressed, enhanced MRI showed venous ectasias within the superficial cutaneous and deeper subcutaneous lesions (Figure 2). Duplex scan ruled out arteriovenous fistula in this malformation. We diagnosed this case as a CM with masked venous malformation and planned for percutaneous sclerotherapy using 1% polidocanol under the guidance of ultrasound.

Sclerotherapy was performed in the outpatient clinic. Ultrasonographic guidance was used before the approach to puncture the lesion. First, the needle tip

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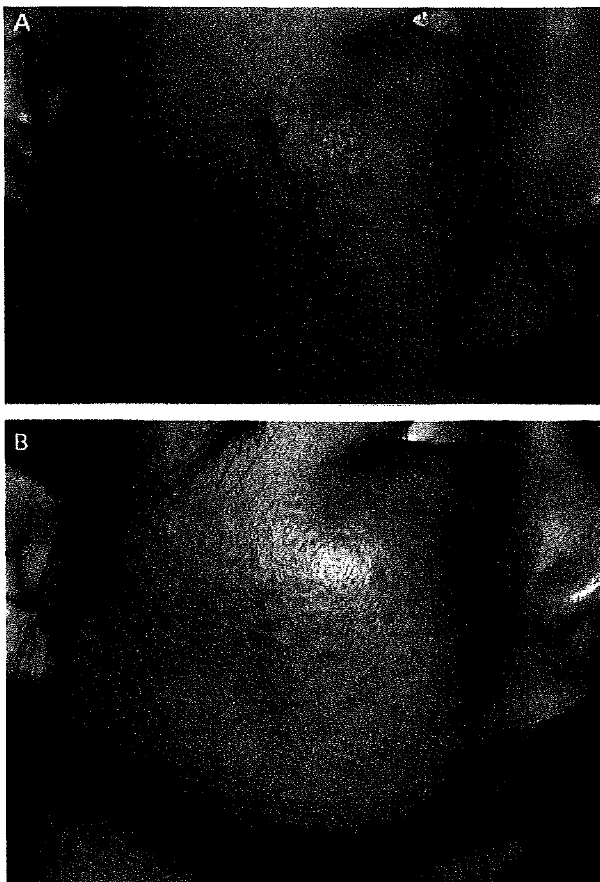

Figure 1. Views before (A) and after (B) percutaneous sclerotherapy. After eight treatment sessions scarring of the venous pools causes a noticeable decrease in the overall size of the lesion, thus achieving the desired cosmetic result.

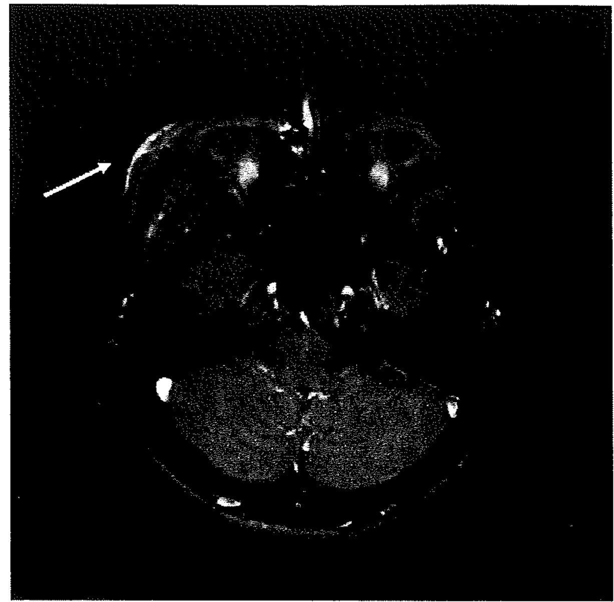

Figure 2. T2-weighted enhanced magnetic resonance imaging with fat suppression shows venous ectasias of superficial cutaneous and subcutaneous lesions.

was guided using ultrasound into the venous cavity. Second, aspiration was performed, and upon visualization of good blood return, as further confirmation of the location of the needle tip within the venous pool, 1% polidocanol (0.5–2.0 mL per treatment session) was slowly infiltrated to ensure that there was no extravasation into normal skin. After eight sclerotherapy sessions, a noticeable decrease in the overall size and discoloration of the lesion could be seen, thus achieving the desired cosmetic result (Figure 1B). After almost 2 years there has been no recurrence.

Discussion

PDL has been the treatment of choice for CMs, but in the majority of cases, complete clearance cannot be achieved, and a significant proportion of lesions are resistant to the treatment. The factors contributing to the poor response have been studied in some previous reports. Fiskerstrand and colleagues and Selim and colleagues investigated the correlations between vascular morphological characteristics and the response to treatment. Subsequently, biopsies revealed that the mean vessel diameter of poorly responding groups were significantly smaller or larger than that of groups with good response. The mean diameter of vessels that responded to PDL was in the range of 16.5 to 51.2 μm.[6,7] Onizuka and colleagues estimated the mean depth for a favorable response to be 930 μm.[8] Anatomic location is a well-known prognostic factor. CMs in dermatome V2 and on the distal limbs show poorer response, because they have more deeply placed vessels.[9]

We evaluated this case with a CM that was resistant to PDL using MRI and duplex ultrasound. The result provided information about the vessel characteristics as well as the subcutaneous extent of the lesion. We found venous ectasias of the superficial cutaneous and deeper subcutaneous components of the lesion.

Therefore, this case was diagnosed as CM with masked venous malformation. We performed percutaneous sclerotherapy using 1% polidocanol.[10] Percutaneous sclerotherapy was developed as a minimally invasive treatment modality and is usually used in venous malformations and macrocystic lymphatic malformations. CMs have not yet been a candidate for sclerotherapy. Superficial lesions with small vascular channels might be difficult to cannulate, even with a small needle, and there would be a high risk for skin necrosis if the sclerosant were to extravasate. In principle, sclerotherapy in the facial region is thought to be dangerous because of possible flow of the drug toward the orbital veins, especially in cases in which the lesions are close to the angular and supraorbital veins, but in this case, the venous malformation was located in the lateral region of the cheek, far from the orbital region. Furthermore, sclerotherapy is performed with careful and slow infiltration, to minimize the risks of the sclerosant flowing to the orbital regions. In cases with a risk of the sclerosant flowing to the orbital regions, the drainer vein should be manually compressed to enhance the safety of the treatment. We performed sclerotherapy without any local anesthesia, infiltrating the polidocanol gradually while carefully observing for any complications. The main reason for choosing polidocanol sclerotherapy under the guidance of ultrasound in this case was that there were venous ectasias in the deeper component of the lesion. In such a case, proper preoperative examination and investigations are required to confirm the diagnosis and to select the most cost-effective therapeutic modality.

Recently, a method of using foam polidocanol has been introduced, and foam sclerotherapy is considered more effective than liquid sclerothetapy in the treatment of venous malformations, even extensive venous malformations.[11,12] Cabrera and co-workers reported that polidocanol foam was effective with intramuscular venous malformations that have small arteriovenous communications.[11] Breu and Guggenbichler described the indications for foam sclerotherapy concerning the sizes of the veins. They reported that the larger the diameter of the vein, the more viscous the foam should be to obtain better results, and liquid foams are probably not sufficiently effective, whereas the smaller the diameter of the vein, the more liquid the foam should be, for easier injection and to reduce possible tissue damage.[13] Guex described that the liquid sclerosing agent pushes and replaces blood in veins smaller than 3 mm in diameter.[14] Although the foam sclerosant can be visualized on ultrasonography, it may occasionally be difficult to reveal the extent of the sclerosant into small-diameter veins because sclerosing foam is highly echogenic. Therefore, we elected to use polidocanol in liquid form for this case.

Percutaneous polidocanol sclerotherapy may be useful in the management of CMs that respond poorly to PDL, especially in patients with venous ectasias of the deeper subcutaneous components of the lesion. Each patient should be evaluated for the permeation of the lesion and be offered laser therapy, percutaneous sclerotherapy, or both to achieve the desired cosmetic result with the least risk of complications.

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Pigment Cell Melanoma Res. 22; 601–610 ORIGINAL ARTICLE

Laminin-421 produced by lymphatic endothelial cells induces chemotaxis for human melanoma cells

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Summary

Melanoma has a high tendency to metastasize to lymph nodes, which is one of the clinicopathological factors to indicate poor prognosis. Recent investigations have shown the importance of lymphangiogenesis in lymph node metastasis in a variety of human tumors including melanoma. However, molecular mechanism of lymphatic metastasis is still poorly defined. We examined influence of interactions between normal lymphatic endothelial cells (LECs) and melanoma cells on cell migration. Medium conditioned with LEC (LEC-CM) contained chemotactic and chemokinetic activities for human melanoma cell lines. The chemotactic activity was fractionated in more than 100 kDa, and inactivated by heat-treatment. The chemotactic activity of LEC-CM was abolished by immunodepletion with anti-laminin-1 antibody. And immunoprecipitation and Western blot analyses revealed that LEC-CM contained laminin-421. When melanoma C8161 cells were treated with function-blocking antibodies to integrin α3 or α6, their chemotactic responses to LEC-CM were markedly reduced. Furthermore, the knock-down of tetraspanin CD151 weakened the chemotactic responses of C8161 and MeWo cells to LEC-CM. These data suggest that laminin-421 secreted by LEC possibly facilitates lymphatic metastasis through the induction of chemotaxis of melanoma cells.

Introduction

Cutaneous melanoma is one of the most aggressive solid tumors, and its incidence and mortality rates are increasing in most countries (Marks, 2000). Aggressiveness of melanoma is characterized by its highly metastatic ability and resistance to chemotherapy and immunotherapy (Gajewski, 2007; Postovit et al., 2006; Satyamoorthy and Herlyn, 2002; Soengas and Lowe, 2003). Cutaneous melanoma metastasizes frequently to

Significance

Cutaneous melanoma is one of the most aggressive solid tumors, and its incidence and mortality rates are increasing in most countries. Regrettably, the molecular mechanism of lymphatic metastasis by melanoma cells remains unclear. We examined the roles of interaction between melanoma cells and LECs, especially the influence of laminins secreted by LECs on the migration of melanoma cells. While the roles of laminins of the basement membrane of blood vessels in invasion and metastasis have actively been researched, their roles in lymphatic vessels remain unclear as it has been difficult to specifically identify lymphatic endothelial cells. Thus, there are only few reports on laminins secreted by LECs. We suspect that laminin-421 secreted by LEC possibly facilitates lymphatic metastasis through the induction of chemotaxis of melanoma cells.

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regional tumor-draining lymph nodes, which is one of the major prognostic factors for tumor recurrence and survival (Balch et al., 2001). Once the melanoma has spread to the lymph nodes, only 40–50% of these patients survive for 5 yr (Tsutsumida et al., 2005). Therefore, sentinel lymph node biopsy is widely performed to determine regional lymph node status in patients with melanoma. However, the molecular mechanism of lymphatic metastasis by melanoma cells remains unclear. Recently, molecular markers of lymphatic endothelial cells (LECs) have been identified, for example, podoplanin, Prox1, and LYVE1 (Hirakawa and Detmar, 2004). Discovery of these markers enables us to histologically identify lymphatic endothelium in intra- and peri-tumor tissues and to establish primary cultured LECs. Studies using LEC markers and cultured LECs indicated the importance of lymphangiogensis in melanoma tissues, particularly a correlation of the number of lymphatic vessels and the incidence of lymph node metastasis (Dadras et al., 2003; Shields et al., 2004). Furthermore, it was demonstrated that vascular endothelial growth factor (VEGF)-C and -D secreted by tumor cells promoted lymphangiogenesis (Skobe et al., 2001; Stacker et al., 2001). It was also reported that CCL21, a chemokine secreted by LECs, stimulated migratory activity of melanoma cells expressing its receptors such as CXCR4 and CCR7 (Fernandis et al., 2004; Muller et al., 2001).

In this study, we examined the in vitro interaction of LECs and human melanoma cells, especially the influence of soluble factors secreted by LECs on the migration of melanoma cells.

Results

Characterization of LECs

Lymphatic endothelial cells (LECs) used in this study were established from vascular endothelial cells by selecting CD31- and podoplanin-positive cells. We first examined the expressions of marker molecules of LEC to confirm that the cells were lymphatic endothelial cells but not those of blood vessels. RT-PCR analyses showed that the LECs expressed podoplanin, Prox-1, and VEGFR-3 (marker genes of LEC) but not CD34 (a marker gene of vascular endothelial cells) (Figure 1A). Immunostaining analysis revealed that all the cells were podoplanin-positive and none of them was positive for CD34 (Figure 1B).

LEC-CM stimulated chemotaxis of melanoma cells

To determine whether LEC-conditioned medium (LEC-CM) stimulates cell motility of melanoma, we carried out chemotaxis assay. As a resultant, LEC-CM induced chemotaxis of all of the melanoma cell lines examined whereas control medium (serum-free EGM-2 MV medium) had no chemotactic activity for any melanoma cell line (Figure 2A). Checkerboard analysis showed that LEC-CM contained both chemotactic and chemokinetic activities for C8161 and MeWo cells although chemokinetic activity was weak compared with chemotactic one (Figure 2B). For heat sensitivity, LEC-CM was incubated at 56°C for 30 min or at 90°C for 5 min. The chemotactic activity of LEC-CM was completely abolished after exposure to 90°C whereas no

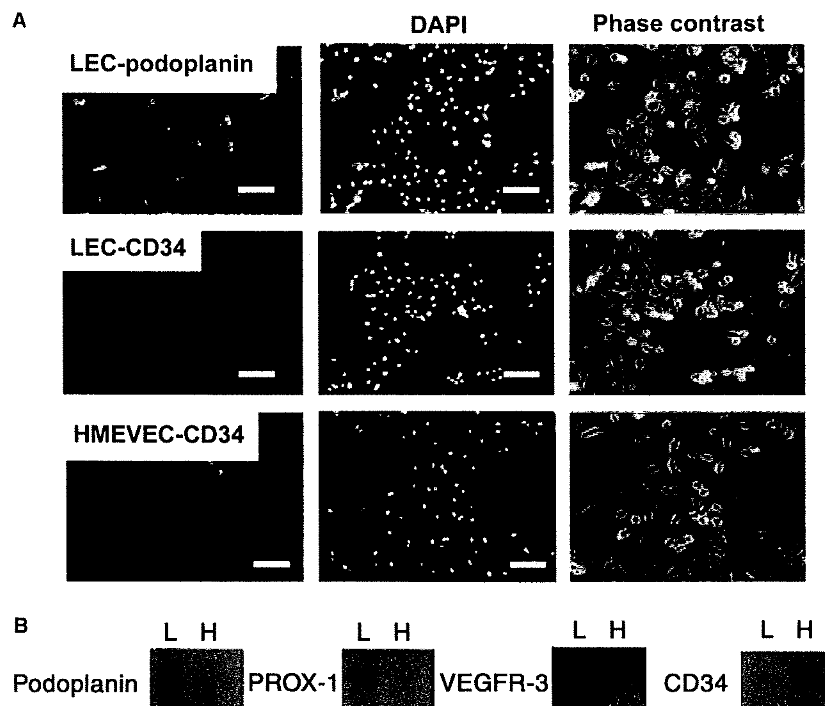

Figure 1. Characterization of lymphatic endothelial cells (LECs). (A) Podoplanin, a specific marker of LECs, and CD34, a specific marker of microvascular endothelial cells, were detected by immunocytochemistry in LECs used in this study and human dermal microvascular endothelial cells (HMVECs). Nuclei were counterstained with DAPI. Bar, 100 μm. (B) Expressions of marker genes of LECs (podoplanin, PROX-1 and VEGFR-3) and vascular endothelial cells (CD34) were detected by RT-PCR using the pair of primers listed in Table 1. L, LECs; H, HMVECs.

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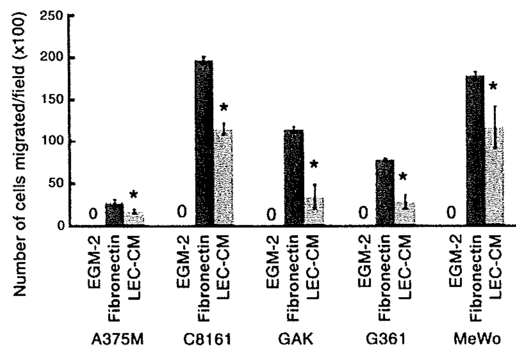

Concentration of LEC-CM in the lower compartment (%)	Number of cells migrated/field (x100) Concentration of LEC-CM in the upper compartment (%)			
	0	25	50	100
0	0 (0)	0 (0)	0 (0)	0 (0)
25	54 (9.27)	29.2 (6.05)	33.4 (4.32)	12.7 (3.34)
50	94.9 (8.60)	37.4 (8.54)	35.6 (4.79)	12.6 (2.50)
100	134.2 (10.56)	80 (11.78)	34.2 (4.16)	35.2 (6.73)

Figure 2. Chemotactic activity of medium conditioned with lymphatic endothelial cells (LEC-CM) for human melanoma cells. Chemotaxis assay was performed by using Transwell chambers. (A) LEC-CM contained chemotactic activity for all the melanoma cell lines. EGM-2 and EGM-2 containing fibronectin (10 μg/ml) were used as a negative and a positive control, respectively. Data are shown as mean ± standard deviation (n = 30). P-values were calculated according to unpaired two-tailed Student's t test: *P < 0.01 compared with the negative control. (B) Checkerboard analysis of C8161 cell migration induced by LEC-CM. Data are expressed as the mean of the migrated cell number per field (n = 20). Numbers in parentheses indicate standard deviation.

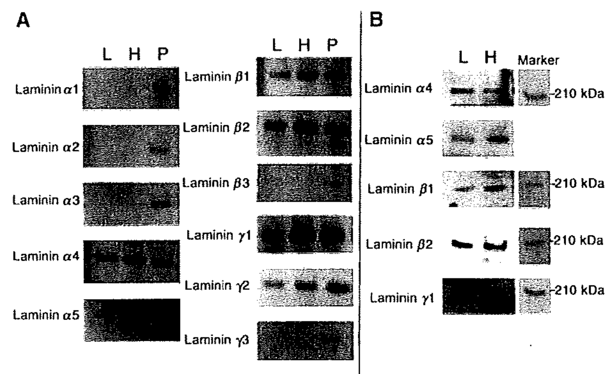

Figure 3. Expression of laminin chains by lymphatic endothelial cells (LECs). (A) Expressions of five α chains, three β chains, and three γ chains were detected by RT-PCR using the pair of primers shown in Table 1. L, LECs; H, HMVECs; P, positive controls – mRNA of human kidney for α1, mRNA of human skeletal muscle for α2, mRNA of human brain for α3 and γ3, mRNA of human placenta for α4 and 5, β1, 2 and 3, and γ2, and mRNA of human small intestine for γ1. (B) Laminin α4 and 5, β1 and 2, and γ1 were detected in LEC-CM by Western blot analysis.

loss was observed in the activity of the 56°C-treated samples (data not shown). In size fractionation of LEC-CM with ultrafilters (MWCO, 30 kDa or 100 kDa), the major chemotactic activity was detected in the crude fraction of more than 100 kDa (data not shown).

Expression of laminin chains in LECs

We explored candidate molecules which were secreted in LEC-CM, of more than 100 kDa and with chemotactic activity, based on the data featured by microarray analysis (Hirakawa et al., 2003; Petrova et al., 2002; Podgrabinska et al., 2002). We focused on components of extracellular matrices, especially laminins which are known to promote cell adhesion and migration (Engbring and Kleinman, 2003; Patarroyo et al., 2002). Laminins are the major component of basement membrane, and composed of three different polypeptide chains, termed α, β, and γ. At present, five α, three β, and three γ chains are known in human (Aumailley et al., 2005; Miner and Yurchenco, 2004). To determine which type of laminin LECs produce, we examined the expressions of 11 laminin chains by RT-PCR. As shown in Figure 3A, LECs expressed laminin α4, α5, β1, β2, γ1, and γ2 chains. Western blot analysis revealed that these laminin chains except γ 2 chain were secreted in LEC-CM (Figure 3B).

Chemotactic activity of LEC-CM was abolished by the treatment with anti-laminin 1 antibody

By the immunodepletion method we examined whether the laminins in LEC-CM induced chemotaxis of melanoma cells or not. For immunodepletion of laminins, we used rabbit anti-laminin1 polyclonal antibody which recognizes epitope localized on γ 1 chain (Champliaud et al., 1996). The chemotactic activity for C8161 cells was almost completely abolished in the LEC-CM immunodepleted with anti-laminin1 antibody (Figure 4A). The immunoprecipitates were subjected to Western blot analysis. As shown in Figure 4B, they contained laminin α4, β2, and γ1 chains, but did not contain laminin α5 and β2 chains. To determine whether laminin α4, β2, and γ1 form heterotrimeric complexes, we carried out SDS-PAGE of LEC-CM in a non-reducing condition and Western blot analysis. The analyses revealed that LEC-CM contained heterotrimeric laminin composed of laminin α4, β2, and γ1 chains (Figure 4C).

Expressions of integrins and CD151

We next analyzed the expressions of laminin-binding integrins such as $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ on the cell surface by flow cytometry. All the melanoma cell lines examined expressed α3, α6, and β1 integirns, but not β4 integrin (Figure 5). We also analyzed the expression levels of tetraspanin CD151 which interacts with the laminin-binding integrins including $\alpha3\beta1$ and $\alpha6\beta1$ and regulates integrin-mediated cellular response (Fitter et al., 1999;

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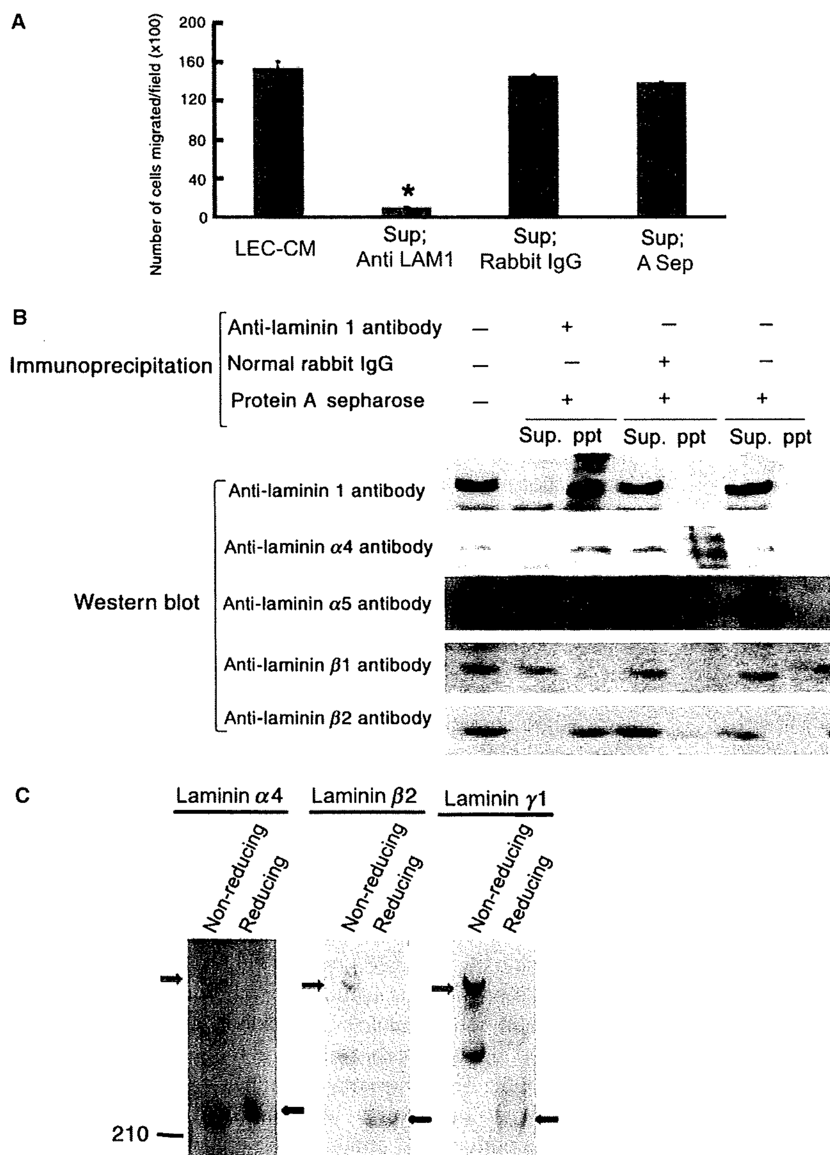

Figure 4. Immunodepletion of chemotactic activity from medium conditioned with lymphatic endothelial cells (LEC-CM) by anti-laminin 1 antibody. (A) Chemotactic activities of LEC-CM for C8161 cells before and after immunoprecipitation with anti-laminin 1 antibody-Protein A Sepharose complex (sup; anti LAM1), normal rabbit IgG-Protein A Sepharose complex (sup; rabbit IgG) or Protein A Sepharose alone (sup; A Sep). Data are shown as mean ± standard deviation (n = 20). *P < 0.01 compared with LEC-CM, sup; rabbit IgG and sup; A Sep by unpaired two-tailed Student's t test. (B) Laminin chains immunoprecipitated from LEC-CM with anti-laminin 1 antibody were detected by Western blot analysis. Immunoprecipitates with normal rabbit IgG and absorbates by Protein A Sepharose were used as negative controls. sup, supernatant of LEC-CM immunoprecipitated; ppt, immunoprecipitates (or materials absorbed by Protein A Sepharose). (C) Proteins contained in LEC-CM were processed for SDS-PAGE in a non-reducing condition or reducing condition, and then analyzed by Western blot using anti-laminin α4, β2 and γ1 antibodies. Gray arrows indicate laminin chains detected in a non-reducing condition. Black arrows indicate laminin α4 (227 kDa), β2 (220 kDa) or γ1 (200 kDa) chain detected in a reducing condition.

Liu et al., 2007; Yang et al., 2008). The expression of CD151 was detected in all the melanoma cell lines (Figure 5).

α3β1, α6β1 integrins and CD151 were implicated in chemotaxis of melanoma cells toward LEC-CM

To determine whether the laminin-binding integrins and CD151 play a crucial role in chemotaxis of melanoma cells toward LEC-CM, we inhibited the function of the integrins and the expression of CD151, respectively. The treatment of C8161 cells with the function-blocking antibodies respectively to integrin α3 (SM-T1) and α6 (GoH3) significantly reduced their chemotactic responses to LEC-CM (Figure 6A, B). To examine the functions of CD151, we performed siRNA gene silencing on CD151 in C8161 and MeWo cells. The transfection of siRNA lowered the expression levels of CD151 in both cell lines (Figure 7A). And the chemotactic activity of the CD151-knocked-down cells toward LEC-CM was significantly reduced compared with that of the control cells (Figure 7B).

Discussion

The mechanisms responsible for lymphatic metastasis of melanoma are poorly defined. In this study, we examined the roles of interaction between melanoma cells and LECs. And we found that laminin-421 secreted by LECs stimulated migration of melanoma cells.

The LEC-CM prepared in our study contained chemotactic activity for human melanoma cells. The chemotactic activity was fractionated in more than 100 kDa. So far, several reports have shown that chemokines such as CCL21 and CXCL12 stimulated chemotaxis of

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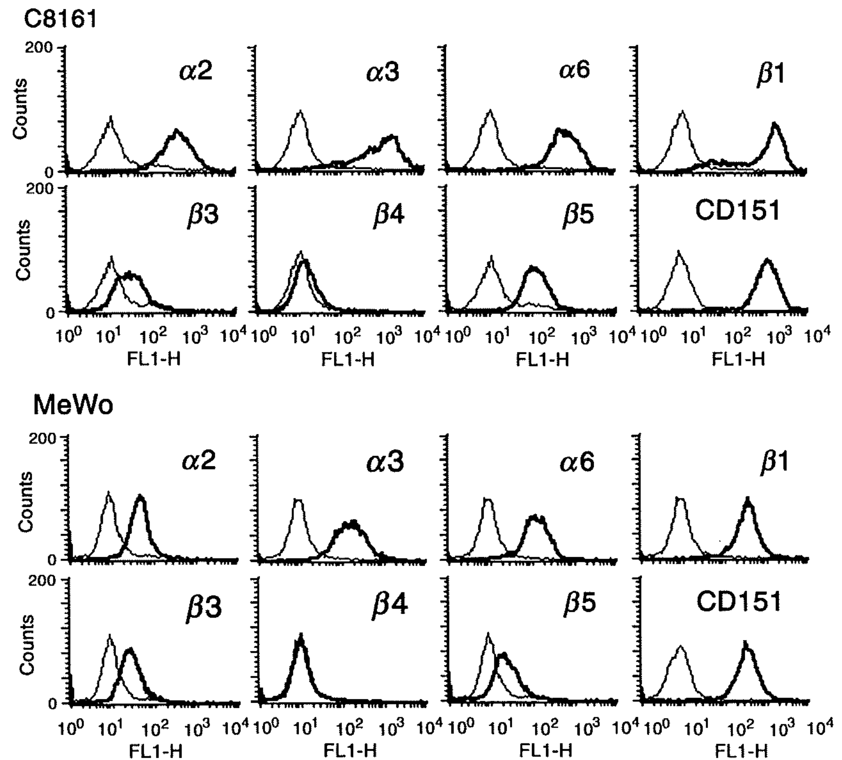

Figure 5. Expressions of integrins and CD151 in human melanoma cells. Expressions of CD151 and integrins including laminin-binding integrins such as $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ on the cell surface of C8161 and MeWo cells were analyzed by flow cytometry. Thin and thick lines indicate fluorescence intensity of cells treated with FITC-conjugated antibody alone and those treated with anti-integrin or CD151 antibodies and FITC-conjugated antibody respectively.

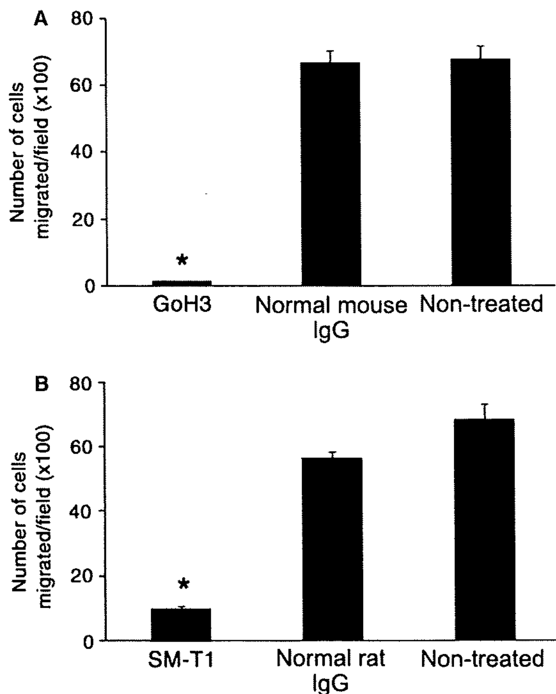

Figure 6. Inhibition of chemotaxis of C8161 cells toward medium conditioned with lymphatic endothelial cells (LEC-CM) by function-blocking anti-integrin antibodies. Treatment of melanoma cells with anti-integrin $\alpha6$ (GoH3, A) or $\alpha3$ (SM-T1, B) antibody started 30 min prior to chemotaxis assay and continued throughout the assay. Data are shown as mean ± standard deviation (n = 30). *$P < 0.01$ compared with C8161 cells treated with or without normal mouse IgG or normal rat IgG by unpaired two-tailed Student's t test.

melanoma cells (Scala et al., 2006; Shields et al., 2004). However, such chemokines are unlikely to be chemotactic factors present in LEC-CM as the molecular weights of those chemokines are too small (*Mr* 8–14 kDa). On the other hand, some microarray analyses have revealed that among the molecules of more than 100 kDa secreted by LECs are the components of extracellular matrix (ECM) such as laminins and nidogen (Hirakawa et al., 2003; Petrova et al., 2002; Podgrabinska et al., 2002). These ECM components are known to promote migratory responses including chemotaxis of various kinds of tumor cells (Engbring and Kleinman, 2003; Lukashev and Werb, 1998). Furthermore, there are several reports indicating that laminins play an important role in invasion of the subendothelial membrane of blood vessels by melanoma cells (Patarroyo et al., 2002; Terranova et al., 1984). Therefore, we focused on the components of extracellular matrix, laminins, and nidogens, as chemoattractants in the LEC-CM.

Nidogen has two isoforms, nidogen-1 and nidogen-2, which function to cross-link between laminins (Takagi et al., 2003). Nidogen-2 is also known to be involved in adhesion mediated by integrin $\alpha3\beta1$ and $\alpha6\beta1$ (Salmivirta et al., 2002). We detected nidogen-1 and -2 in LEC-CM by Western blot analysis (Saito, N., Hamada, J., Furukawa, H., Tsutsumida, A., Saito, A., Moriuchi, T. and Yamamoto, Y., unpublished data). And chemotactic activity of LEC-CM for melanoma cells was not changed by the immunodepletion using neutralizing antibodies to nidogen-1 and/or nidogen-2 from LEC-CM (unpublished data). As the finding indicated that nidogens in LEC-CM

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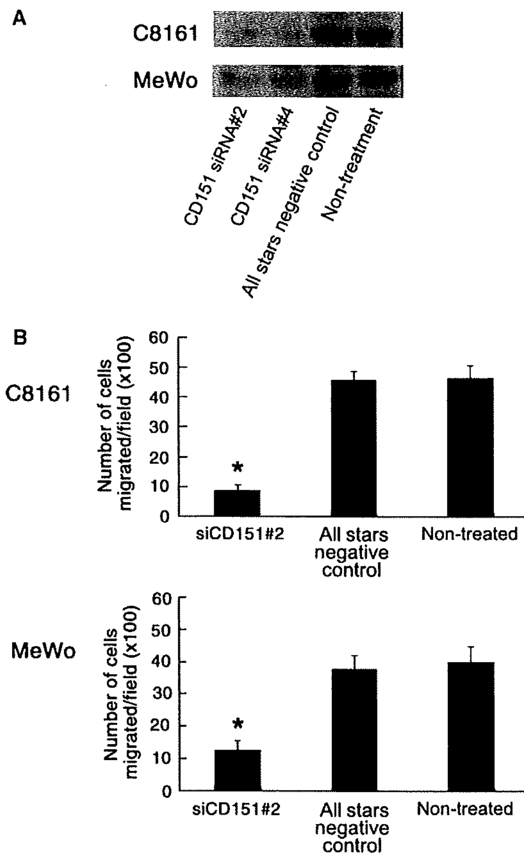

Figure 7. Inhibition of chemotaxis of melanoma cells toward medium conditioned with lymphatic endothelial cells (LEC-CM) by knock-down of CD151. (A) C8161 and MeWo cells were transfected with siRNAs to CD151 (CD151#2 and CD151#4) or control siRNA (AllStars Negative Control). Proteins extracted from the transfectants and non-transfectants were subjected to Western blot analysis. (B) Chemotaxis of C8161 and MeWo cells transfected with or without siRNAs (CD151#2 or AllStars Negative Control). Data are shown as mean ± standard deviation (n = 20), respectively. *$P < 0.01$ compared with melanoma cells transfected with the control siRNA and the non-transfected cells by unpaired two-tailed Student's t test.

did not serve as chemoattractant for melanoma cells, we next focused on laminins. Laminins are a large family of heterotrimeric ECM glycoproteins, which are localized mainly in the basement membrane. They consist of three different subunits, the α, β, and γ chains. To date, five α, three β, and three γ chains, and at least 15 isoforms with various combinations of α, β, and γ chains have been identified (Aumailley et al., 2005; Miner and Yurchenco, 2004). Of the three kinds of laminin subunit chains, the α-chain possesses the binding domain to laminin receptors such as integrins, and is distributed in a tissue-specific pattern (Hallmann et al., 2005).

While the roles of laminins of the basement membrane of blood vessels in invasion and metastasis have actively been researched, those of lymphatic vessels in invasion and metastasis remain unclear as it has been difficult to specifically identify LECs. Thus, there are only few reports on laminins secreted by LECs. Hirakawa et al. (2003) performed comparative gene array analyses of cultured vascular endothelial cells and LECs, and revealed the expression of laminin $\beta1$ and $\beta2$ chains in LECs. Vainionpaa et al. (2007) found laminin $\alpha4$, $\beta2$ and $\gamma1$ chains in lymphatic vessels by immunohistochemistry. In this study, we found that LECs produced laminin $\alpha4$, $\alpha5$, $\beta1$, $\beta2$, and $\gamma1$ chains. The chemotactic activity of LEC-CM for melanoma cells was abolished when laminins including $\gamma1$ chain were depleted by anti-laminin-1 antibody which had previously been reported to recognize human $\gamma1$ chain (Champliaud et al., 1996). Furthermore, the immunoprecipitate of LEC-CM with anti-laminin-1 antibody contained laminin $\alpha4$, $\beta2$, and $\gamma1$ chains. And Western blot analysis in a non-reducing condition demonstrated that LEC-CM contained laminin heterotrimer composed of $\alpha4$, $\beta2$, and $\gamma1$ chains (laminin-421). These data indicated that LEC-CM secreted laminin-421 which induced chemotaxis of melanoma cells. In the experiment shown in Figure 4, laminin $\alpha5$ chains were not detected in immunoprecipitates with anti-laminin-1 antibody which had previously been demonstrated to recognize human $\gamma1$ chains (Champliaud et al., 1996). However, the affinity and specificity of the antibodies used such as anti-laminin-1 antibody and anti-laminin $\alpha5$ chain antibody have not fully been characterized and it is difficult to evaluate the absolute amounts of $\alpha4$ and $\alpha5$ chains in LEC-CM. Furthermore, as it was evident that $\alpha5$ chains were produced by LEC (Figure 3B), we consider that heterotrimeric complexes with $\alpha5$ chains such as laminin-511 or –521 were theoretically contained in LEC-CM. Thus, we cannot eliminate the possibility that heterotrimeric laminins containing $\alpha5$ chains may have functioned as chemoattractants for melanoma cells besides laminin-421 which must have been the major source of chemotactic activity in LEC-CM.

If melanoma cells migrated toward laminins in LEC-CM, they should express the receptors for laminins such as adhesion molecules which belong to integrin family. It is known that integrin $\alpha3\beta1$ and $\alpha6\beta1$ bind to laminin $\alpha4$ or $\alpha5$ chain (Vainionpaa et al., 2007). As expected, all the melanoma cells used here expressed these integrins on their surfaces. To determine whether the chemotaxis of C8161 cells toward LEC-CM was dependent on integrin $\alpha3\beta1$- or $\alpha6\beta1$-mediated signals, we blocked the function of each integrin with antibody SM-T1 or GoH3. As a resultant, the treatment with each antibody significantly inhibited chemotaxis of C8161 cells to LEC-CM. Tetraspanin CD151 assembles laminin-binding integrins to make a complex called tetraspanin-enriched microdomains, and controls integrin-dependent cell spreading, migration, and adhesion (Fitter et al., 1999; Liu et al., 2007; Yang et al., 2008). For examining possible involvement of CD151 in the chemotaxis of melanoma cells toward LEC-CM, the expression of

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CD151 was knocked-down in C8161 and MeWo cells by the transfection with siRNA to CD151. CD151 knock-down weakened the chemotactic response of both cell lines to LEC-CM. These results strongly suggest that LECs produce laminin-421 to which melanoma cells migrate in integrin $\alpha3\beta1$- and/or $\alpha6\beta1$-dependent fashion.

Lymphatic vascular system plays critical roles not only in the immune response but also in tumor metastasis. While, it still remains obscure how tumor cells migrate across the lymphatic endothelium and form invasive growth in lymph nodes (Pepper and Skobe, 2003). This study may help us to understand the mechanism of invasion of lymphatics in primary lesions and lymph nodes by aggressive tumors including melanoma.

Materials and methods

Cells and culture condition

Human A375M and C8161 melanoma cells were kindly provided by Dr. Saiki (Institute of Natural Medicine, Toyama University, Toyama, Japan) and Dr Nakajima (Johnson and Johnson, Tokyo, Japan), respectively. GAK cells were obtained from Institute for Fermentation (Osaka, Japan) (Nozawa et al., 1984). G361 cells were obtained from Riken Cell Bank (Tsukuba, Japan). MeWo cells were obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). The melanoma cells were grown on tissue culture dishes in a 1:1(v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (DME/F12) supplemented with 10% of fetal bovine serum (FBS). Human LECs, which were established from neonatal microvascular endothelial cells by purification method using immunobeads coated with anti-CD31 antibody and anti-podoplanin antibody, were purchased from Angio Bio (Del Mar, CA, USA). Lymphatic endothelial cells were maintained in EGM-2 MV medium (Lonza, Walkersville, MD, USA). The cell lines were cultured in a CO_2 incubator (5% CO_2 and 95% air).

We confirmed the expression patterns of the genes specific to LECs, namely, podoplanin, PROX-1, and VEGFR-3. Each pair of primers for RT-PCR is shown in Table 1. For immunocytochemistry, LECs were fixed in 10% formalin for 30 min and exposed to mouse monoclonal anti-podoplanin antibody (Table 2) or mouse monoclonal anti-CD34 antibody (Table 2) for 1 hr at room temperature. After washing, the cells were incubated with biotinylated anti-mouse IgG (Chemicon, Temecula, CA, USA) for 45 min at room temperature, and then with avidin-conjugated Alexa 488 (Molecular Probe, Eugene, OR, USA) for 45 min at room temperature. After staining the nuclei with DAPI (1 μg/ml, Dojindo, Kumamoto, Japan) for 30 min at room temperature, the cells were observed under a fluorescence microscope.

Table 2. Antibodies used in this study

Antigen	Antibody	Reference/Source
Podoplanin	18H5	Angel et al., 2007; abcam
CD34	BI-3C5	Torsney et al., 2007; abcam
Laminin α4	V-20	Kim et al., 2005; Santa Cruz
Laminin α5	H-160	Virtanen et al., 2000; Santa Cruz
Laminin β1	4E10	Wewer et al., 1983; Chemicon
Laminin β2	22	Paroni et al., 2002; BD Biosciences
Laminin γ1	2E8	Cauli et al., 2004; Chemicon
Laminin 1	polyclonal	Durbeej et al., 1996; Sigma
CD151	11G5a	Zheng and Liu, 2006; Acris
Integrin α2	AK7	Favaloro et al., 1990; Chemicon
Integrin α3	ASC-1	Pattaramalai et al., 1996; Chemicon
Integrin α3	SM-T1	Tsuji et al., 2002
Integrin α6	4F10	Woods et al., 1986; Chemicon
Integrin α6	GoH3	Sonnenberg et al., 1987; BioLegend
Integrin β1	mAb13	Akiyama et al., 1989; Becton Dickinson
Integrin β3	RUU-PL7F12	Metzelaar et al., 1991; Becton Dickinson
Integrin β4	ASC-3	Zahir et al., 2003; Chemicon
Integrin β5	AST-3T	Ohta et al., 2006; Frontier Science

LEC-conditioned medium

When LECs (passage numbers 3–4) plated on culture dishes reached 80–90% confluency, the medium was discarded; the monolayer cultures were washed twice with serum-free EGM-2 MV medium and then incubated with serum-free EGM-2 MV medium for 24 hr. The medium was collected and centrifuged at 800*g*

Table 1. Primers for PCR analysis in this study

Gene	Primer sequence (5′–3′) Forward	Primer sequence (5′–3′) Reverse	Size of product (bp)
Podoplanin	tgatgtggtgactccaggaa	cgatggctagtaagacccca	283
PROX-1	cagcccgaaaagaacagaag	tccggttgtaaggagtttgg	354
VEGFR-3	cgctcccaacagacccacac	caaactcggtccaggtgtcc	684
CD34	tccagctgtgcggagtttaagaa	tgctgaatggccgtttctggaggt	536
Laminin α1	taactggtggcaaagtcccagc	cggcaaggctcatcctcataag	915
Laminin α2	aaggctgcgatgagtgtttctg	ggataggagacagcggattcaag	598
Laminin α3	gctctgactgatgcagataactcgg	tctatccacggtggtctgaattg	906
Laminin α4	aaggagcagtggccgact	ctgcttctgggacggactt	360
Laminin α5	gctccaaacttccggtgac	tttcatcaccgctagccg	403
Laminin β1	tgcctcagtacacctcctctgatag	cgtcacatctggaaccaatgtatcc	819
Laminin β2	tgcgagcagtgtcaggatttc	tcagttccaactctgcccattg	961
Laminin β3	aaatggggggaaggtccaact	ccggttccggaaatagtgca	547
Laminin γ1	ggaagctgagaatctggaaca	atcagctcgggctaggagtt	157
Laminin γ2	ccagtgcaaagcaggctacttc	aggcaggaatgttagtgtttccc	581
Laminin γ3	ctccgaggaatgcacgtttga	tggtggaaatcgctgaggatg	522
GAPDH	tgaaggtcggagtcaacggatttggt	catgtgggccatgaggtccaccac	983

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for 10 min and the supernatants were filtered through 0.2 μm filters and stored at −80°C until use.

Chemotaxis assay

Chemotaxis assay was performed by using Transwell chambers (Costar, Cambridge, MA, USA) with 6.5-mm diameter, tissue culture-treated filters with 8 μm pores, according to our previous report (Hamada et al., 1992). Melanoma cells (5×10^5/ml) were suspended in serum-free EGM-2 MV medium, and the cell suspensions (100 μl) were then placed into the upper compartment of chambers. Six-hundred micro liter of LEC-CM, serum-free EGM-2 MV medium containing 10 μg/ml of bovine plasma fibronectin (Sigma, St. Louis, MO) (as a positive control) or serum-free EGM-2 MV medium (as a negative control) was placed into the lower compartment. After incubation for 6 hr, the cells were fixed in 5% glutaraldehyde solution and stained with Giemsa solution. After the cells attached to the upper side of the filters (non-migrated cells) were removed by wiping with a cotton swab, those attached to the lower side of the filters were counted under a microscope. Chemotactic activity was evaluated by the number of the cells per field at ×100 magnification (mean ± standard deviation, n = 20 or 30).

Temperature stability and size fractionation of LEC-CM

For temperature stability studies, LEC-CM was incubated at 56°C for 30 min or at 90°C for 5 min. For crude size fractionation, LEC-CM was fractionated by using a 30 kDa or 100 kDa molecular weight cut-off (MWCO) ultrafilter (Microcon-30 or -100; Millipore, Billerica, MA, USA) by centrifugation at 4°C for 10 min until no fluid was visible in the upper chamber. The retentate (more than 30 kDa or 100 kDa MW fraction) was reconstituted to the original volume with serum-free EGM-2 MV medium.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from monolayer cultures of LECs with Trizol. For reverse transcription (RT), 3 μg of total RNA was subjected to cDNA synthesis in 10 μl of reaction mixture containing First-Strand Buffer (Invitrogen, Carlsbad, CA, USA), 200 U/μl of Moloney murine leukemia virus reverse transcriptase (Invitrogen), 0.1 mM Random Primers (Invitrogen, Carlsbad, CA), 0.1 M dithiothreitol (Invitrogen) and 2.5 mM dNTP Mix (Applied Biosystems, Foster City, CA, USA). The RT reaction was performed sequentially for 2 hr at 37°C and for 10 min at 70°C. PCR amplification of cDNA was performed in 20 μl of reaction mixture containing 1 μl of cDNA sample, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM dNTP Mix, 5 U/μl AmpliTaq DNA Polymerase, and 0.1 mM of primers. Cycling conditions in terms of the number of cycles and annealing temperature were optimized for each pair of primers (Table 1). cDNA of the GAPDH gene was used as an internal control. PCR conditions were as follows: 95°C for 10 min, 28–32 cycles; at 94°C for 1 min, for 40 s at appropriate annealing temperature, 40 s at 72°C for 10 min. PCR products were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

Western blot analysis

Medium conditioned with lymphatic endothelial cells was concentrated to 10 times with Microcon-100. Extraction of proteins from monolayer cultures was as follows. The cells were washed twice with chilled phosphate buffered saline, and then harvested with a cell scraper in lysis buffer (10 mM Tris-HCl pH7.5, 1% NP40, 0.15 M NaCl, 1 mM EDTA, 5 μg/ml Pepstatin, 5 μg/ml Leupeptin, 5 μg/ml Aprotinin). The cell lysates were centrifuged at 20 000*g* for 30 min at 4°C, and the supernatants were collected. The concentrated LEC-CM (2.5 μg) or proteins extracted from the cells (10 μg) were subjected to SDS-PAGE (5–10% gradient gel) in reducing or non-reducing condition and then electrotransferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membranes were blocked at room temperature for 1 hr with 5% skim milk and 0.5% bovine serum albumin in TBS-T buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% Tween-20). The blocked membrane was incubated with primary antibodies for 1 hr at room temperature. The primary antibodies used were listed in Table 2. The membranes were incubated next with horseradish peroxidase-conjugated antibodies for 1 hr at room temperature, and developed by the Immobilon Western Chemiluminescent HRP Substrate System (Millipore).

Immunoprecipitation of laminin

Medium conditioned with lymphatic endothelial cells concentrated in the same manner as described above was incubated with rabbit anti-laminin1 polyclonal antibody (Table 2) bound to Protein A-Sepharose beads (GE Healthcare, Uppsala, Sweden) at 4°C overnight. After centrifugation at 1500*g* for 1 min, supernatant was collected, and then the beads were washed three times and ressuspended in the sample buffer for SDS-PAGE.

Flow cytometry analysis

Flow cytometry analysis was performed to examine the expressions of integrins and CD151 on cell surface. The cells were suspended in phosphate buffered saline (PBS) containing 0.065% sodium azide. They were incubated with primary antibodies for 1 hr at 4°C and then with secondary antibodies conjugated with fluorescein isothiocyanate (FITC) for 45 min at 4°C. After each incubation, the cells were washed thrice with PBS containing 0.065% sodium azide. The stained cells were resuspended in 1 ml of PBS containing 0.065% sodium azide and analyzed with FACSCalibur (Becton Dickinson, San Jose, CA, USA). The primary antibodies used were listed in Table 2. The secondary antibodies used were FITC-conjugated goat anti-mouse IgG (Cappel, Aurora, OH, USA) and FITC-conjugated goat anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Blocking the functions of integrin α3 and α6

Rat anti-integrin α3 antibody (SM-T1, Table 2) was prepared as described in our previous report (Tsuji et al., 2002), and mouse anti-integrin α6 antibody (GoH3, Table 2) was purchased from Bio Legend (San Diego, CA, USA). SM-T1 (30 μg/ml) or GoH3 (1 μg/ml) was added into cell suspension (2×10^5/ml) 30 min prior to chemotaxis assay. Chemotaxis assay was performed in the presence of each blocking antibody in the upper compartments of Transwell chambers.

Inhibition of human CD151 expression by small interfering RNA (siRNA)

C8161 cells (5×10^5) were seeded on 60-mm tissue culture dishes 16 hr before transfection. They were transfected with 20 pM of each siRNA with the use of Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions. The sequences of siRNA used were siCD151-#4 (sense: 5′-GCAGGUCUUUGGCAUGA-3′; antisense: 5′-U CAUGCCAAAGACCUGCUU-3′) and siCD151-#2 (sense: 5′- CCUCAA GAGUGACUACAUCUU-3′; antisense: 5′-GAUGUAGUCACUCUU GAGGUU-3′) Thermo Fisher Scientific, Waltham, MA, USA. AllStars Negative siRNA conjugated with Cy3 was used as the negative control siRNA (Qiagen, Germantown, MD, USA). About 24 h after the transfection, transfection efficiency was checked by monitoring AllStars Negative siRNA conjugated with Cy3 under a fluorescence

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microscope. And then we performed Western blot analysis to confirm repression of CD151 and chemotaxis assay.

Statistical analysis

Results were expressed as mean values and the standard deviation. Statistical analysis was performed by using an unpaired two-tailed Student's t test when comparing two groups. $P < 0.01$ was considered as significant.

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LETTER TO THE EDITOR

MICROSURGERY 29:499–501 2009

USE OF THE ARCADE VESSELS AFTER DISRUPTION OF THE VASCULAR PEDICLE OF PEDICLED JEJUNUM TRANSFER FOR A RECURRENT ESOPHAGEAL CANCER PATIENT

Dear Editor,

As an esophageal replacement for the reconstruction after subtotal esophagectomy (in thoracic esophageal cancer), the first choice is the gastric tube. However, the stomach sometimes cannot be used due to a prior gastrectomy or the coincidence of gastric disorder.[1] In such cases, the jejunum, the colon, and tubing of cutaneous or fasciocutaneous flap can be employed as a substitute for the gastric tube. This case report describes a patient in whom a pedicled jejunum transfer survived by anastomosing arcade vessels after disruption of the vascular pedicle of pedicled jejunum for the recurrence esophageal cancer on postoperative 2 years.

A 67-year-old man presented with a T3N1M0 squamous cell carcinoma of the thoracic esophagus. He had undergone partial gastrectomy due to gastric cancer 14 years ago. He underwent chemotherapy (5-fluorouracil, *cis*-platinum) and radiotherapy (60Gy/30fr) as a primary treatment. One year after the chemoradiotherapy, the tumor appeared at the distal original site and he underwent esophagectomy and resection of gastric remnant. The reconstruction of esophagus has been done with the pedicled jejunum in subcutaneous route. Although he underwent recurrent tumor resection of peritoneal cavity 2 months after the esophagectomy, the tumor rerecurred in the peritoneal cavity a year and 8 months after the latest operation. When he was referred to our department, the tumor was suspected to invade mesentery of jejunal graft and involve vessels of jejunal graft examined by CT.

At the operation, the arcade vessels that were the feeding vessels of jejunal graft were severed because the tumor invaded mesentery of jejunal graft and involved arcade vessels (Figs. 1a and 1b). At this point, vascular impairment of the jejunal graft was observed. We were going to perform vascular reconstruction using arcade vessels since the jejunal segment distal to the severance had a dark color due to interruption of the blood supply. At first, we have carefully dissected and exposed the arcade vessels for vascular anastomosis from mesentery under microscope, and the next, the proximal stump of the jejunal arcade artery and vein were anastomosed to distal stump of that in an end-to-end manner by 9-0 nylon under microscope (Figs. 2a and 2b). The distal segment of the jejunal graft had a good color after microvascular anastomosis. After that, redundant jejunum with poor vascularity between the proximal and distal stump was excised. We could monitor the flap survival easily by seeing peristalsis of grafted jejunum in subcutaneous route and assisted by endoscope. He was suffered from sepsis postoperatively; however, he has improved by treatment of antibiotics. The patient was allowed fluids by mouth on postoperative day 21. The patient was discharged from hospital on postoperative day 65. Three-month postoperatively, he can eat regular food.

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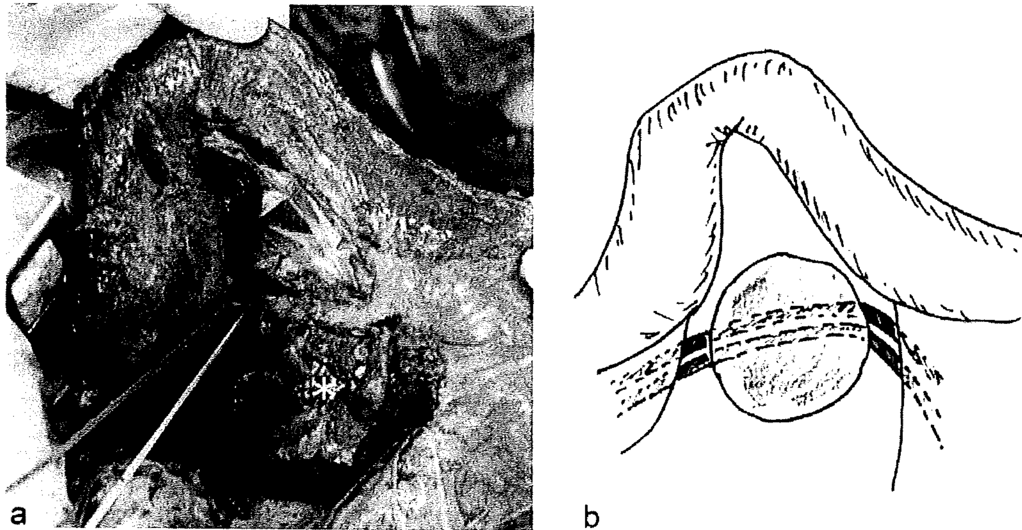

*Figure 1. Intraoperative findings (**a**) and schematic diagram (**b**) before excision of tumor. The tumor (·) involved arcade vessels of pedicled jejunal graft.*

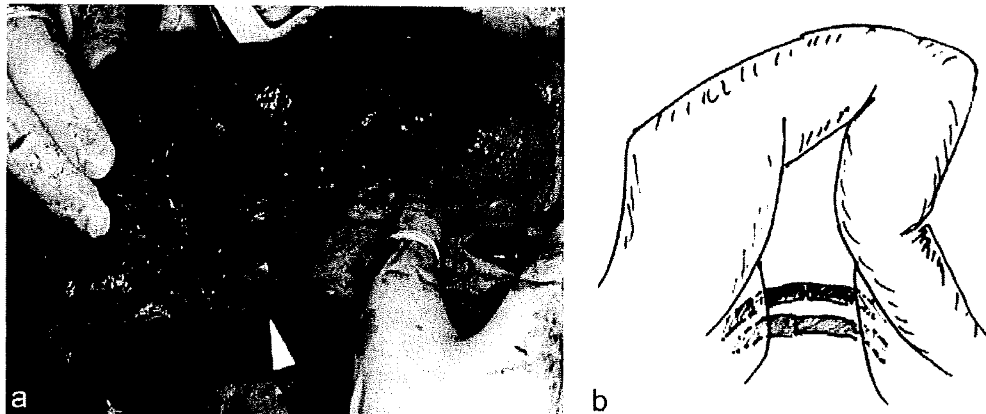

*Figure 2. Intraoperative findings (**a**) and schematic diagram (**b**) after microvascular anastomoses. The proximal and distal stamp of the arcade vessels was anastomosed. The distal jejunal segment had a good color after microvascular anastomoses.*

Although the pedicled jejunum transfer, not free jejunum transfer, had been performed for this patient, we consider that disruption of vascular supply in the pedicled graft is similar to that in the free graft, because small intestine has a single dominant axial blood supply, with no collateral blood supply from peripheral tissues.[2,3] Several authors reported the survival of free jejunal transfers after late or immediate interruption of blood supply.[4–6] It is possible that revascularization may take longer or may never occur in the cases of small intestine graft, because the intestine is fed by a single inherent arterio–venous system, which is suspended in the peritoneal cavity, and the serous membrane may act as a sort of barrier when intestine is grafted and may delay its acceptance.[2,3]

In this case, we have performed direct anastomoses of arcade vessels since it was possible for jejunum graft to result in necrosis after disruption of vascular supply. Although we have experienced little difficulty in dissection and exposure of the arcade vessels from scar tissue, esophageal rereconstruction was performed successfully without making other donor site morbidity.

Although we have described only a case and supporting data are lacking, we consider that the technique of vascular anastomoses using arcade vessels contributed to perfusion of distal segment jejunal graft and deceased to ischemic complications.

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D. 悪性腫瘍：下肢

2. 下腿　遊離皮弁

山本有平

〈術者からのコメント〉

穿通枝皮弁の概念は，当初，筋・腱間中隔に存在する穿通血管を茎として挙上する皮弁として認識されていたが，現在では各種筋肉を貫く筋内穿通血管を茎とする皮弁へと広がっている．欠損部の位置や大きさにより，本皮弁を遊離皮弁として用いている症例も多い．本ケースは，深下腹壁動静脈穿通枝皮弁を遊離皮弁として移植した下腿再建症例である．本皮弁は，仰臥位で大きな皮弁が採取でき，また太く長い血管柄をもち，さらに脂肪部分を切除することにより皮弁の厚さの調節が可能であり，下肢の悪性皮膚軟部組織腫瘍切除後の整容的再建に適している[1]．

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1) 山本有平　他：各種穿通枝皮弁を用いた下肢悪性腫瘍の治療経験．Skin Cancer 18：334-337，2003

症例：60歳，男性（図1～4）．
診断名：悪性線維性組織球腫（右下腿部伸側）
現症：7×8×3cmの皮下腫瘤
画像所見：MRIでは右下腿外側前面に充実性の腫瘤性病変を認め，前脛骨筋，長趾伸筋，長腓骨筋に広く接しており浸潤の可能性を示唆した．胸・腹部・骨盤CT，ガリウムシンチグラフィーにて，リンパ節や他臓器への転移を示唆する所見は認めなかった．

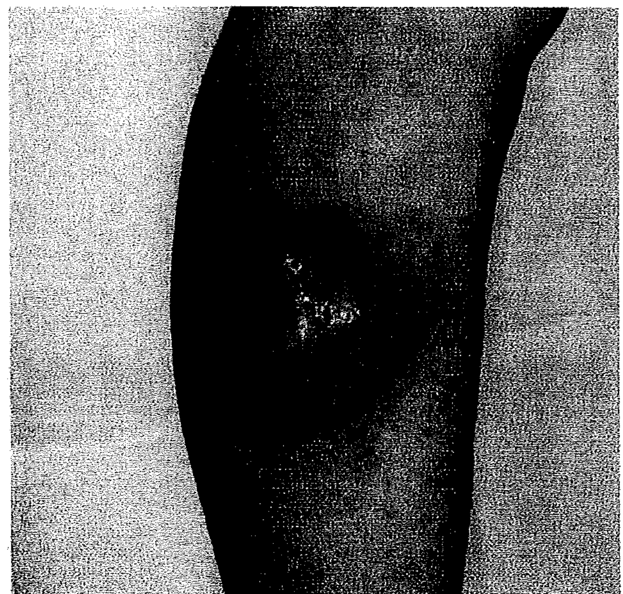

図1：術前．部分生検のマーキング．右下腿部の悪性組織球腫．

治療：部分生検により，悪性の線維組織球性の軟部組織腫瘍と診断されている．病変部辺縁より周囲3cm離し，深部は前脛骨筋，長趾伸筋，長腓骨筋および腓腹筋とヒラメ筋の一部を含めて腫瘍を切除し，一部骨が露出した．広範囲切除後に生じた欠損部は13×14cmであった．再建に用いた皮弁は，左傍臍～下腹部に位置する深下腹壁動静脈穿通枝を血管茎として，13×17cmの大きさでデザインした．皮弁を腹直筋前鞘上で挙上し，2本の穿通枝を確認後，腹直筋を切離して深下腹壁動静脈を血管柄とする遊離皮弁として採取した．皮弁の皮下脂肪を部分的に切除し，皮弁の厚さのthinningを行った．右前脛骨動静脈をレシピエント側の吻合血管とし，動脈を端側吻合，伴行静脈2本を端端吻合した．

経過：術後経過は良好で皮弁は完全生着した．病理組織学的診断は悪性線維性組織球腫であり，断端部はすべて陰性であった（stage IIB; G2T2 by Enneking's staging system, wide excision）．

本症例は参考文献[1]にて発表しており，図を引用している．

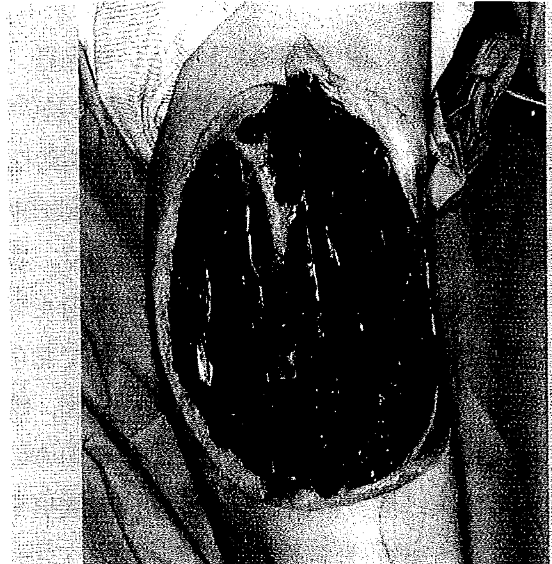

図2：欠損部．一部，骨と神経が露出している．

図3：皮弁挙上．深下腹壁動静脈を血管茎としている．

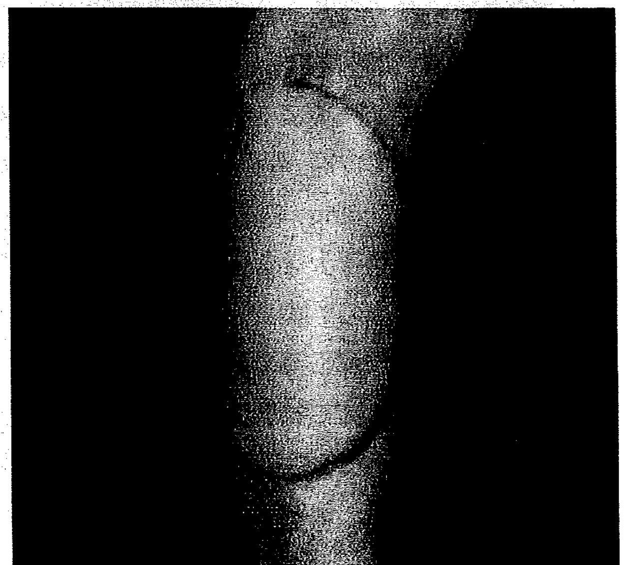

図4：術後1年．良好に再建されている．