Monoclonal Antibody to Very Late Antigen-4 (VLA-4) Protects Skin Flaps against Ischemia-Reperfusion Injury: An Experimental Study in Rats

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Abstract: In recent years, various adhesion molecules have been discovered. Very late antigen-4 (VLA-4), an adhesion molecule belonging to the integrin family, plays an important role in lymphocyte migration and infiltration into sites of inflammation. In the present study, we investigated whether administration of anti-VLA-4 monoclonal antibody (mAb) reduces inflammation in ischemia-reperfusion injury using a skin flap model in Sprague-Dawley rats. A superficial epigastric arteriovenous pedicle flap, sized 45×30 mm, was elevated in the right inguinal area. The vessel was clamped to induce ischemia and the clamp was removed after 9 hours. Fifteen minutes prior to clamp release, the rats were administered anti-VLA-4 mAb (0.2 mg/kg i.v., n = 10) or saline (n = 10). At the same time, a sham group (n = 5) was established with a similar operation but the vessel was only clamped to induce ischemia for 5 minutes. Anti-VLA-4 mAb significantly improved the survival area of the skin flap to 92.4% (median), when compared to 12.5% (median) in the control group. Severe inflammatory cell infiltration and edema was consistently observed in the skin flaps of the control group, while anti-VLA-4 reduced these parameters to a level similar to the sham group. These results indicate that anti-VLA-4 mAb may be used as an anti-inflammation agent, however the underlying mechanism still remains to be elucidated.

Key words: ischemia-reperfusion injury, very late antigen-4 (VLA-4), adhesion molecule, anti-VLA-4 monoclonal antibody, skin flap

Introduction

In recent years, various adhesion molecules have been discovered. Such molecules are closely associated with cellular interactions and play crucial roles in inflammation and the regulation of immunoreaction1). They play an important role in the progress of inflammation by activating leucocyte adherence to vascular endothelial cells. The tissue infiltration process of an inflammatory reaction can be classified into the following three steps: rolling step, strong adhesion step, and transmigration step. In each step, different adhesion molecules are involved.
The phenomenon called “ischemia-reperfusion injury” is known to play an important role in the mechanism of skin flap survival and necrosis. In particular, it has been suggested that changes in vascular endothelial cells during ischemia-reperfusion injury are involved in preventing skin flap survival and causing necrosis\(^2\)\(^-\)\(^6\). To elucidate the mechanisms involved in skin flap survival and necrosis, a rat model of skin flap ischemia-reperfusion injury has been developed. Thus, we used this rat model to understand the causative agents responsible for inflammation\(^6\),\(^7\).

Integrin, one of the adhesion molecules, is known to be highly involved in the regulation of intercellular and extracellular matrix adhesion and intracellular signal transduction via adhesion\(^8\). Very late antigen-4 (VLA-4), a member of the integrin family, is an adhesion molecule that plays an important role in the migration and infiltration of lymphocytes to sites of inflammation\(^9\). Therefore, we investigated whether the administration of anti-VLA-4 monoclonal antibody (mAb) reduces the inflammatory reaction in ischemia-reperfusion injury using the rat skin flap model. Here we report the results, along with a review of the current literature.

**Materials and methods**

*Skin flap ischemia-reperfusion injury model*

Twenty-five male Sprague-Dawley rats (8–10 weeks of age, 225–250 grams, Saitama Experimental Animals Supply Co., Ltd., Saitama, Japan) were used in this experiment. The animals were housed in wire-mesh cages and given free access to a pelleted diet (Nosan Corporation, Kanagawa, Japan) and drinking water (tap water).

Ketamine hydrochloride (25 mg/kg) was administered intraperitoneally as anesthesia. While anesthetised, the abdomen was shaved and a 45×35 mm superficial epigastric pedicled skin flap was designed and elevated from the right inguinal region. The flap pedicle was then clamped (Fig. 1)\(^6\). The animal experiment was conducted ethically according to the Showa University animal experiment guidelines.

*Ischemic interval*

The duration of ischemia was 9 hours for the anti-VLA-4 mAb and control groups, or 5 minutes for the sham group.

*Antibody administration*

Anti-rat VLA-4 mAb (0.20 mg/kg; CD49d; TA-2, Associates of Cape Cod Incorporated, Falmouth, MA) was administered through the caudal vein 15 minutes before releasing the clamp. Sterilized saline was administered to the control group.

*Experimental groups*

The anti-VLA-4 mAb experimental group (n = 10) and the physiological saline control group (n = 10) were observed for 7 days. The same skin flap was elevated in the sham group (n = 5), however the duration of ischemia was only 5 minutes. All rats were euthanized after 7 days of observation by an overdose of anesthesia, and histological examination of the skin flaps was
performed (Fig. 2).

Method of evaluation

Skin flaps were evaluated by macroscopic observation and histological analysis.

1) Skin flap survival area ratio: the areas of the survival region and necrotic region of the skin flap were calculated by tracing the areas, based on the template method of Morris et al\textsuperscript{10}, and the survival area ratio was calculated as \( \frac{\text{survival area}}{\text{survival area} + \text{necrotic area}} \times 100 \).

2) Histological examination: samples were prepared by hematoxylin-eosin staining. Tissue samples were then examined to assess the extent of inflammation, and inflammation within blood vessels.

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**Fig. 1.** A: Diagram of the skin flap model (flap size, 45×35 mm) showing the primary vessels and the points where they were occluded (see reference #6).

**Fig. 2.** Protocol for anti-very late antigen-4 (VLA-4) monoclonal antibody administration in ischemia-reperfusion injury of the rat skin flap
Statistical analysis

The data are expressed as median, with 25th and 75th percentiles. A Mann-Whitney U test was applied to evaluate the difference in survival area ratio between control and treatment groups. Differences of $P < 0.05$ were considered to be statistically significant.

Results

Skin flap survival area ratio

A nonparametric analysis of the experimental group and the control group was performed. Macroscopically, skin flap survival in the experimental group was good, and skin flap morphology and color were close to the sham group. However in the control group, skin flaps showed severe inflammation accompanied by partial necrosis. The mean skin flap survival area ratio after 7 days observation was 92.4% in the experimental group, which was significantly improved compared to 12.5% in the control group ($P < 0.05$; Fig. 3 and Table 1).

![Fig. 3. Viability of skin flaps in each group](image)

Left panel: Scatterplot of survival area ratio (%) of skin flaps in the sham (n = 5), anti-very late antigen-4 monoclonal antibody (VLA-4 mAb; n = 10), and control (n = 10) groups after ischemia-reperfusion injury. Ischemia time was 9 hours in VLA-4 mAb and control groups, and 5 minutes in sham group. Horizontal lines represent median, 25th and 75th percentiles. $P < 0.05$, VLA-4 vs. control.

Right top: Representative picture from the sham group on Day 7, showing a survival area ratio of 100%. Right middle: Representative picture from the anti-VLA-4 mAb administration group on Day 7, showing a survival area ratio of 100%. Some skin flaps in this group showed approximately 10% necrosis at the periphery, but on median, the survival rate was 92.4%.

Right bottom: Representative picture from the saline-treated control group on Day 7, showing the entire skin flap is necrotic. On median, the survival area ratio for this group was 12.5%.
Histological examination

In the experimental group, although inflammation was present and a disorganized structure was observed on part of the skin surface, the level of inflammatory cell infiltration and edema was relatively mild, and the vascular lumen was preserved, with a tissue structure similar to that of the sham group. In the control group, inflammatory cell infiltration, edema and necrosis were observed. In addition, the vascular lumen was filled with erythrocytes, indicating the formation of blood clots, which was dramatically different from the condition of the vascular lumen in the experimental group (Fig. 4).

Table 1. Survival area ratio of skin flaps after 9 hours ischemia-reperfusion injury

|                       | VLA-4 mAb | Control | P-value |
|-----------------------|-----------|---------|---------|
| No. of rats           | 10        | 10      |         |
| Survival area ratio (%) |          |         |         |
| Median                | 92.4      | 12.5    | 0.0009  |
| 25th, 75th percentiles | 50, 100   | 0, 37   |         |

VLA-4 mAb, very late antigen-4 monoclonal antibody.

Fig. 4. Histological sections from representative skin flaps
A: Section from the sham-operated group showing normal tissue structure after only 5 minutes of ischemia.
B: Slight edema is noted in the papillary dermis with moderate inflammatory infiltrate in skin flaps treated with anti-very late antigen-4 monoclonal antibody after 9 hours of ischemia.
C: In control skin flaps receiving saline vehicle, after 9 hours of ischemia, the epidermis is necrotic and the dermis shows edema with significant inflammatory infiltration composed of lymphocytes. Hematoxylin-eosin staining. Magnification×100.
Discussion

VLA-4 is also known as integrin alpha 4 beta 1, CD49d/CD29, or lymphocyte Peyer’s patch HEV adhesion molecule 2. VLA-4 is expressed in T-cells, B-cells, and hematocytes, such as monocytes, natural killer cells, eosinophils, neutrophils, basophils, thymocytes, myelomonocytes, erythroid progenitors, and lymphoid progenitors, along with melanomas and muscle cells.

Activated VLA-4 attaches onto vascular endothelial cells, and is a fibronectin receptor. Unlike typical fibronectin receptors, such as VLA-5, the binding of VLA-4 with fibronectin is not via the RGD sequence. VLA-4 induces migration of lymphocytes and monocytes to the site of inflammation by binding with inflammation-induced vascular cell adhesion molecule-1 (VCAM-1) on vascular endothelial cells. Then, the binding of VLA-4 with VCAM-1 leads to both rolling and adhesion of leukocytes. In short, it is believed that activated lymphocytes and macrophages expressing VLA-4 partially facilitate extravascular migration by binding with VCAM-1 which is expressed on the vascular endothelial cell by inflammatory cytokines.

Various adhesion molecules expressed on activated leukocytes and vascular endothelial cells are involved in ischemia-reperfusion injury. Previously, in an experimental skin flap model of ischemia-reperfusion injury, inflammation was significantly suppressed by the administration of anti-intercellular adhesion molecule-1 mAb. In the present study, we found that the administration of anti-VLA-4 mAb significantly suppressed the inflammation induced by ischemia-reperfusion injury. VLA-4 is involved with the rolling, adhesion and extravascular migration of leukocytes by binding with VCAM-1, which is expressed on vascular endothelial cells during inflammation. It is suggested that anti-VLA-4 antibody blocks the adhesion binding of VLA-4 with activated VCAM-1 on vascular endothelial cells and leukocytes during inflammation, consequently the rolling step is blocked, resulting in suppression of inflammation.

Kerrigan and Stotland suggested that in ischemia-reperfusion injury, platelet activating factor, interleukin-6, and tumor necrosis factor (TNF) are induced after reperfusion to promote tissue damage. Moreover, there are several studies reporting that the administration of platelet activating factor antagonist or tacrolimus significantly reduces ischemia-reperfusion injury. Inhibition of neutrophil-induced superoxide radical formation, inhibition of neutrophil-induced mediators such as TNF or leukotriene B4, suppression of reperfusion-induced TNF or interleukin-6, and attenuation of the neutrophil-vascular endothelial cell binding interaction by suppression of CD11/CD18 activity have been mentioned as possible mechanisms involved in ischemia-reperfusion injury.

Experimentally, it has been revealed that VLA-4 is related to the development of multiple sclerosis, based on the mouse model of autoimmune encephalomyelitis. Additionally, it has been suggested that VLA-4 plays a role in cellular differentiation in bone marrow, as well as an important involvement in infiltration and metastasis of kidney cancer and acute myeloid leukemia. Consequently, there has been a clinical study demonstrating the suppression of inflammation by administration of the anti-VLA-4 human mAb, natalizumab.

Conclusion

In this study, we found inflammation was reduced by the administration of anti-VLA-4 mAb in a rat inguinal skin flap ischemia-reperfusion injury model. Anti-VLA-4 mAb significantly improved the survival area of the skin flap up to 92.4%, compared to 12.5% in control group. These results suggest that anti-VLA-4 mAb can be used as an anti-inflammation agent to prevent ischemia-reperfusion injury.

Conflict of interest disclosure

There are no conflicts of interest to declare concerning this study.

References

1) Springer TA. Adhesion receptors of the immune system. Nature. 1990;346:425-434.
2) Marzella L, Jesudass RR, Manson PN, et al. Functional and structural evaluation of the vasculature of skin flaps after ischemia and reperfusion. Plast Reconstr Surg. 1988;81:742-750.
3) May JW Jr, Chait LA, O’Brien BM, et al. No-reflow phenomenon in experimental free flaps. Plast Reconstr Surg. 1978;61:256-267.
4) Eriksson E, Replogle RL, Glagov S. Reperfusion of skeletal muscle after warm ischemia. Ann Plast Surg. 1987;18:224-229.
5) Lee C, Kerrigan CL, Tellado JM. Altered neutrophil function following reperfusion of an ischemic myocutaneous flap. Plast Reconstr Surg. 1992;89:916-923.
6) Tosa Y, Lee WPA, Kollias N, et al. Monoclonal antibody to intercellular adhesion molecule 1 protects skin flaps against ischemia-reperfusion injury: an experimental study in rats. Plast Reconstr Surg. 1998;101:1586-1594; discussion 1595-1596.
7) Yokoyama T, Tosa Y, Fujimaki A, et al. The effect of FK506 (Tacrolimus) against ischemia-reperfusion injury in rats. J Jpn Soc Plast Reconstr Surg. 2000;20:108-113. (in Japanese).
8) Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell. 1992;69:11-25.
9) Alon R, Kassner PD, Carr MW, et al. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. J Cell Biol. 1995;128:1243-1253.
10) Morris SF, Pang CY, Zhong A, et al. Assessment of ischemia-induced reperfusion injury in the pig latissimus dorsi myocutaneous flap model. Plast Reconstr Surg. 1993;92:1162-1172.
11) Wayner EA, Garcia-Pardo A, Humphries MI, et al. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J Cell Biol. 1989;109:1321-1330.
12) Mould AP, Komoriva A, Yamada KM, et al. The CS5 peptide is a second site in the CS region of fibronectin recognized by the integrin alpha 4 beta 1. Inhibition of alpha 4 beta 1 function by RGD peptide homologues. J Biol Chem. 1991;266:3579-3585.
13) Bednarczyk JL, McIntyre BW. A monoclonal antibody to VLA-4 alpha-chain (CDw49d) induces homotypic lymphocyte aggregation. J Immunol. 1990;144:777-784.
14) Osborn L, Hession C, Tizard R, et al. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell. 1989;59:1203-1211.
15) Tosa Y, Hosaka Y, Satoh K. A skin flap model for ischemia-reperfusion injuries. Jpn J Plast Reconstr Surg. 2005;48:1005-1012. (in Japanese).
16) Kerrigan CL, Stotland MA. Ischemia reperfusion injury: a review. Microsurgery. 1993;14:165-175.
17) Steinman L. Blocking adhesion molecules as therapy for multiple sclerosis: natalizumab. *Nat Rev Drug Discov.* 2005;4:510–518.

18) Tomita Y, Saito T, Saito K, et al. Possible significance of vLA-4 (alpha 4 beta 1) for hematogenous metastasis of renal cell cancer. *Int J Cancer.* 1995;60:753–758.

19) Sanchez-Madrid F, De Landazuri MO, Morago G, et al. VLA-3: a novel polypeptide association within the VLA molecular complex: cell distribution and biochemical characterization. *Eur J Immunol.* 1986;16:1343–1349.

20) Rice GP, Hartung HP, Calabresi PA. Anti-alpha 4 integrin therapy for multiple sclerosis: mechanisms and rationale. *Neurology.* 2005;64:1336–1342.

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