Reduction of Ischemia-reperfusion Injury to Skin Flap by Treatment with Monoclonal Antibody to Endothelial Leukocyte Adhesion Molecule-1

Masakazu Ohashi, Yasuyoshi Tosa, Yoshiaki Hosaka
Kaneshige Satoh, Mehmet Oguz Yenidunya and Baohua Pang

Abstract: The selectin family, which mediates adhesion of activated leukocytes to the vascular endothelium, plays an important role in inflammation. Endothelial leukocyte adhesion molecule-1 (ELAM-1) is expressed on activated vascular endothelium and mediates the special leukocyte rolling phenomenon seen when leukocytes adhere to vascular endothelium. We investigated changes in skin flap survival/necrosis due to treatment with the monoclonal antibody (Mab) to ELAM-1. Twenty-five male Sprague-Dawley rats (225-250 g) were used in the experiment. A 45 × 30 mm pedicled skin flap with superficial epigastric vessels was designed in the right inguinal region and prepared. Animals in the treated group received anti-ELAM-1 Mab intravenously 15 minutes prior to reperfusion; those in the control group received normal saline. By tracing the outline of viable and nonviable areas skin flap viability was assessed. Data were collected for the subsequent 7 days. These data corroborated with histological evidence on comparable areas of the flap. Tracing analysis revealed an average flap survival area of 91.7% in the treated group and 18.3% in the control group (P < 0.005). Histopathologically, few inflammatory changes were observed in the treated group, while marked damage was observed in the control group. We conclude that treating skin flaps with anti-ELAM-1 was effective in reducing ischemia-reperfusion (I-R) injury after 9 hours of warm ischemia.

Key words: ischemia-reperfusion injury, flap, selectin family, endothelial leukocyte adhesion molecule-1 (ELAM-1), monoclonal antibody to ELAM-1

Introduction

Changes to vascular endothelial cells in ischemia-reperfusion injury are one factor which prevents the survival of skin flaps and causes necrosis. Tosa et al have developed a rat ischemia-reperfusion injury skin flap model and are investigating the role of a variety of cytokines in the survival/necrosis of skin flaps. This rat ischemia-reperfusion injury skin flap model is useful for determining what causes inflammation and for devising potential treatments.
A variety of cell adhesion molecules have been discovered and their functions clarified\(^{12}\). The adhesion of activated leukocytes to vascular endothelial cells plays an important role in the progress of inflammatory reactions\(^{12}\). The process of tissue infiltration by leukocytes occurs in three stages: rolling, adhesion, and transmigration. Further, each process involves a different adhesion molecule. The first-stage, rolling phenomenon, mainly involves the selectin family.

Endothelial leukocyte adhesion molecule-1 (ELAM-1) is one member of the selectin family which is expressed on endothelial cells activated by cytokines and involved in the adhesion to neutrophils. ELAM-1 is involved in the leukocyte rolling phenomenon that occurs at the initial stage of inflammation and is also involved in myocardial ischemia-reperfusion\(^{13,14}\).

It is reported that inflammation can be decreased in an ischemia-reperfusion injury skin flap model by using antibodies to the adhesion molecules ICAM-1 (intercellular adhesion molecule-1) and LECAM-1 (leukocyte endothelial cell adhesion molecule-1)\(^{10,11}\). In this study we investigated the skin flap survival ratio and tissue changes when administrating anti-ELAM-1 monoclonal antibodies using this ischemia-reperfusion injury skin flap model.

**Material and Methods**

1. **Ischemia-reperfusion injury skin flap model**

Twenty-five male Sprague-Dawley rats (8-10 weeks of age, 225-250 grams) were used. The animals were housed in wire-mesh cages and given free access to pellet diet and drinking water. Ketamine hydrochloride (25 mg / kg) was administered intraperitoneally as anesthesia. The abdomen was shaved and 45 × 35 mm pedicled skin flaps with superficial epigastric veins and arteries as pedicles were designed in the right inguinal region and elevated. Then the skin flap pedicles were clamped (Fig. 1\(^{5}\)). The animal experiment was conducted ethically following the Showa University animal experiment guidelines.
2. Ischemic interval
The ischemic interval was 9 hours, or 5 minutes for the sham group.

3. Antibody administration
An anti-ELAM-1 antibody (0.20 mg/kg) (AF977, R&D Systems Inc., Minneapolis, MN, USA) was administered by intravenous injection to the caudal vein 15 minutes before releasing the clamp. Sterilized saline at the same dose was administered in the same manner as the control.

4. Experimental groups
An anti-ELAM-1 antibody treatment group (n = 10) and a physiological saline control (n = 10) were observed over 7 days. A skin flap was elevated in the same manner in the sham group (n = 5), but the clamp was released after 5 minutes. The animals were euthanized at the end of the 7-day observation by an overdose of anesthesia and histological examination of the skin flaps was conducted (Fig. 2).

5. Method of evaluation
Skin flaps were evaluated by macroscopic observation and histological analysis.
1) Skin flap survival area ratio
The area ratio of the skin flap survival and necrotic regions was calculated by tracing based on the template method of Morris et al to obtain the survival area ratio.\textsuperscript{15}

2) Histological examination
Tissue samples were stained with hematoxylin-eosin and observed for characteristics of inflammation, the extent of inflammation and for the condition within blood vessels.

6. Statistical analysis
The data were expressed as mean ± SD. Mann-Whitney statistical analysis was applied to evaluate survival difference between control and treatment groups. Differences of $P < 0.05$ were considered to be statistically significant.
Results

1. Skin flap survival area ratio

Non-comparative analysis of the treatment group administered anti-ELAM-1 antibody and the control group administered physiological saline was conducted. Comparison of macroscopic findings showed that skin flap survival in the treatment group was good with skin flap morphology and color similar to the sham group. However, in the control group, skin flaps showed severe inflammation accompanied by partial necrosis (Fig. 3).

When comparing the mean skin flap survival area ratio in each group on day 7 after reperfusion, administration of anti-ELAM-1 improved the skin flap survival ratio with a statistically significant difference ($P < 0.005$) at $91.7 \pm 12.8\%$ in the antibody group compared to $18.3 \pm 19.6\%$ in the control group (Fig. 4).

2. Histological examination

Although characteristics of inflammation were observed in the treatment group, the degree of inflammatory cell infiltration and edema was comparatively mild, the intravascular lumen was intact and tissue structure close to that of the sham group. In the control group, however, inflammatory cell infiltration, edema and necrosis were observed, showing markedly different findings from the treatment group (Fig. 5).
Reduction of I-R Injury by Mab to ELAM-1

Fig. 4. Percentage survival area of the skin flaps in each group.
Animals in the sham-operated group were ischemic for 5 minutes.
The viable area treated with monoclonal antibody to ELAM-1 was significantly
higher than the viable area of the flap in the control group.

Fig. 5. Histological sections from representative flaps. Hematoxylin-eosin stain (×100)
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 flaps treated with monoclonal antibody to ELAM-1 after 9 hours of ischemia.
C : In control flaps receiving saline vehicle, the epidermis was necrotic and the dermis
showed edema with significant inflammatory infiltration composed of lymphocytes.
Discussion

ELAM-1 is also known as E-selectin or CD62E\textsuperscript{13,14}. Its molecular structure is that of a type I membrane glycoprotein with an extracellular N-terminal region consisting of a lectin domain, EGF (epidermal growth factor) domain and 6 complement regulatory protein (CRP) domains, a membrane-spanning segment and an intracellular domain containing the C-terminal (Fig. 6). Normally, ELAM-1 expression on vascular endothelial cells is low. However, expression is induced upon stimulation by endotoxins or cytokines\textsuperscript{13,14}.

ELAM-1 binds to its ligand via the lectin-like domain on the N-terminal. Known ligands are polysaccharides such as sLe\textsubscript{x} (sialyl Lewis x) and sLe\textsubscript{a} (sialyl Lewis a)\textsuperscript{16}. It also binds with low affinity to PSGL-1, a ligand of P-selectin\textsuperscript{16}. \textit{In vitro} expression on vascular endothelial cells peaks 4-6 hours after stimulation by cytokines such as IL-1\textbeta or TNF\alpha (tumor necrosis factor\alpha) or LPS (lipopolysaccharide), and expression quickly ends about 12 hours after stimulation. The level of expression differs depending on the vascular bed, due to differences in the stability of mRNA\textsuperscript{17}.

The importance of ELAM-1 is mainly its involvement in the rolling of neutrophils, and some lymphocytes, to activated vascular endothelium and the subsequent transition to adhesion of these cells\textsuperscript{18}. Selectins bind weakly to its ligand on a partner cell. As a result a stress gradient is produced between selectin and blood flow and leukocytes roll over the vascular endothelium. Following selectin shedding, chemokines produced by vascular endothelial cells activate the leukocytes, which bind to integrin-mediated intercellular adhesion molecule-1 (ICAM-1). This mediates stronger intercellular adhesion. Finally the leukocytes pass through the intercellular space and infiltrate the extravascular inflammation site (Fig. 7)\textsuperscript{19}.
Ischemia-reperfusion injury is an inflammatory reaction and therefore involves the events described above. An experimental model of ischemia-reperfusion injury using skin flaps was used to clarify this mechanism. In this model, administration of anti-ICAM-1 monoclonal antibodies markedly suppressed the effects of inflammation.

In this study, inflammation was markedly suppressed by monoclonal antibodies against
ELAM-1. It is surmised that administration of anti-ELAM-1 antibody inhibits the adhesion of activated leukocytes to vascular endothelium during the initial stages of inflammation. Thus, the subsequent rolling phenomenon is blocked and inflammation is attenuated (Fig. 8).

Platelet activating factor (PAF), IL-6 and tumor necrosis factor (TNF) are induced after reperfusion in ischemia-reperfusion injuries and the destruction of tissue progresses. Administration of PAF antagonists or tacrolimus reduces the effects of ischemia-reperfusion injury. Several mechanisms of action have been suggested. These include inhibition of superoxide radical formation by neutrophils, inhibition of mediators induced by neutrophils such as TNF and LTB4, inhibition of TNF or IL-6 induction by reperfusion, and weakening of the binding interaction between neutrophils and vascular endothelial cells by inhibition of CD11/CD18. It is further reported that anti-LECAM-1 antibody significantly reduces inflammatory effects in skin flaps.

In experiments on cerebral ischemia, expression of ELAM-1 begins during ischemia, and increases gradually. Expression is also seen in the non-ischemic side after 24 hours. The continuous stimulating effects of post-ischemic cytokines carried to the non-ischemic side by blood flow are thought to account for this phenomenon. Therefore it can be postulated that the inhibition of ELAM-1 immediately before ischemia-reperfusion by anti-ELAM-1 antibodies in our experimental system brought about the marked difference in skin flap survival in the control group and treatment group.

Recently the usefulness of ELAM-1 for inflammatory disorders, and platelet coagulation-related disorders and as a blood marker for vascular endothelial damage has been investigated because its expression is accompanied by the activation of leukocytes, platelets and vascular endothelium due to cytokine or antigen stimulation. Further, cancer cells adhering to vascular endothelium by ELAM-1 are thought to metastasize and to be deeply involved in blood-borne metastasis. In the future it may be possible to apply an index of skin flap necrosis by measuring serum ELAM-1 in an ischemia-perfusion injury skin flap.

In the present experiment a significant reduction in inflammatory effects in skin flaps was observed in the anti-ELAM-1 antibody treatment group in an ischemia-reperfusion injury skin flap model. Further experimental research will be needed for full elucidation of the function of ELAM-1 and the mechanism of action of its monoclonal antibody. However, the marked attenuating effect on inflammation obtained in this research suggests that administration of the monoclonal antibody to ELAM-1 is potentially an effective technique to prevent partial necrosis of skin flaps.

Acknowledgements

This work was supported in part by the Grant-in Aid for Scientific Research (C) (2) No.15591901 from the Ministry of Education, Science, Sports, and Culture of Japan.

Some of these data were presented in part at the 12th Research Council Meeting of Japan Society of Plastic and Reconstructive Surgery, Tokyo, October 9-10, 2003.

References


