Effects of AMD3100 subconjunctival injection on alkali burn induced corneal neovascularization in mice

Liang-Hong Peng¹, Wei Shen², Wang Yong¹, Lu Lu¹, Lin Liu²

¹Department of Ophthalmology, General Hospital of Guangzhou Military Command of Chinese PLA, Guangzhou 510010, Guangdong Province, China
²Department of Ophthalmology, the First Affiliated Hospital of Second Military Medical University, Shanghai 200433, China

Correspondence to: Lin Liu. Department of Ophthalmology, the First Affiliated Hospital of Second Military Medical University, Shanghai 200433, China. linliu@sh163.net

Received: 2010-12-10 Accepted: 2011-02-02

Abstract

- AIM: To investigate the therapeutic effects of local and systemic administration of AMD3100 for alkali burn induced corneal neovascularization (CNV) in mice.
- METHODS: CNV was induced in vivo by alkaline burn of cornea in C57BL/6 mice. AMD3100 was administrated topically by subconjunctival injection or systemically by intraperitoneal injection for 7 days; balanced salt solution was administrated topically or systemically as a control respectively. Inflammatory index was evaluated by slit-lamp biomicroscopy and inflammatory cells infiltrated to cornea tissue were detected by histologic analysis at multiple time points. CNV was compared between the local and systemic treated mice 2 weeks after alkali burn, as quantified by CD34 immunostaining. Fluorescence-Activated Cell Sorter Analysis was used to investigate the mobilizing effects of EPC in mice after subconjunctival injected or intraperitoneal injected AMD3100. Immunohistochemistry was used to detect the expression of endothelial progenitor cells (EPC) marker proteins VEGFR2 and CD34.
- RESULTS: Three days after alkali burn, infiltration of inflammatory cells was found in corneal tissue. At the first 7 days of local injection group, the number of inflammatory cells was significantly lower than that in systemic injection group. CNV could be seen at the 7th day, and at the 14th day reached the peak, then started to decrease. The number of CNV in the subconjunctival injection group was 16.34±1.53 per 0.034mm² vs 13.26±1.87 per 0.034mm². The research also showed that intraperitoneally, but not subconjunctivally injected AMD3100 could mobilize EPC. On the other hand, subconjunctival, but not intraperitoneally injected AMD3100 could reduce the expression of EPC marker proteins.
- CONCLUSION: In mice locally administrated AMD3100 can reduce the number of alkali burn induced CNV. The number of inflammatory cells and inflammatory responses in corneal tissue.
- KEYWORDS: alkali burn; corneal neovascularization; AMD3100; inflammatory cells; EPC

INTRODUCTION

As a pathological response to ischemia and inflammation, postnatal neovascularization (also known as neoangiogenesis) includes the up-regulation of angiogenic growth factors, migration of inflammatory cells, remodeling of the ischemic tissue environment, and the sprouting of new blood-vascular networks. This process was originally considered to result exclusively from the proliferation, migration and remodeling of preexisting endothelial cells. However, the discovery of adult endothelial progenitor cells (EPC) has changed this point of view. In 1997, Asahara et al. firstly showed that CD34+, which came to be known as EPC, cells isolated from adult peripheral blood could differentiate in vitro into endothelial cells and contribute to neoangiogenesis in vivo. Further studies showed that these cells were of bone marrow origin and were able to contribute to both physiologic and pathologic neovascularization. Recent evidence demonstrates that the chemokine stromal-cell derived factor-1 (SDF-1, also known as CXCL12) has a major role in the recruitment and retention of CXCR4+ BM cells to the neoangiogenic niches supporting revascularization of ischemic tissue and tumor growth. SDF-1 is one member
of the chemokines family, which include four groups, CXC, CX3C, CC, and C (C=cytine and X=any amino acid). SDF-1 binds to a high affinity receptor, CXC chemokine receptor 4 (CXCR4). CXCR4 was initially cloned as an orphan chemokine receptor and activated by only one ligand, SDF-1. Previous studies showed that CXCR4 was central to the retention of HPCs within the bone marrow, and perturbation of the CXCR4:CXCL12 chemotactic interaction within the BM results in immobilization. AMD3100 was a specific antagonist of CXCR4 and AMD3100 injection can induce a rapid and potent mobilization of EPC. Monocytes mobilized into the blood by G-CSF or AMD3100 stimulate angiogenesis at sites of ischemia through a paracrine mechanism. Corneal neovascularization (CNV) can not only threaten vision, but also endanger the normal immune privilege status of anterior chamber and increase the risk of graft rejection. However, the exact mechanism that modulating the formation of CNV is not clear. Given that the cornea is normally an avascular tissue and EPC can produce SDF-1 and express SDF-1 receptor CXCR4, we raise a hypothesis that EPC may play an important role in this process, and breaking SDF-1/CXCR4 axis with AMD3100 can affect CNV formation.

MATERIALS AND METHODS

Materials Male, 8-week-old C57BL/6 mice were purchased from the Center of Animals of The Second Military Medical University, Shanghai, China. All animal studies were approved by the Animal Research Committee of The Second Military Medical University and were done according to the guidelines of National Institutes of Health. CNV was induced by alkali burn, as described previously with modified. Briefly, mice were anesthetized with chloral hydrate (5mg/kg) and topical proparacaine. A 0.2-mm disc with modified. Briefly, corneal microvascular were highlighted by immunostaining with anti-CD34 antigen monoclonal antibody (Santa Cruz, CA, USA) in a 1:200 dilution and incubating at room temperature for 2 hours. Any single brown-stained cell or cluster of endothelial cells that was clearly separate from adjacent microvessels and other connective tissue elements was considered as vessel. Branching structures were counted as a single vessel unless there was a discontinuity in the structure. The stained sections were screened at 5 magnification to identify the areas of the highest vascular density within the cornea. Vessels were counted in the 5 areas of highest vascular density at 250 magnification (0.034mm² per field). Microvascular density was expressed as the mean number of vessels in these areas. The counts were performed in a masked manner.

Microvascular density count The methods of microvascular density counting were as described previously. Briefly, corneal microvascular were highlighted by immunostaining with anti-CD34 antigen monoclonal antibody (Santa Cruz, CA, USA) in a 1:200 dilution and incubating at room temperature for 2 hours. Any single brown-stained cell or cluster of endothelial cells that was clearly separate from adjacent microvessels and other connective tissue elements was considered as vessel. Branching structures were counted as a single vessel unless there was a discontinuity in the structure. The stained sections were screened at 5 magnification to identify the areas of the highest vascular density within the cornea. Vessels were counted in the 5 areas of highest vascular density at 250 magnification (0.034mm² per field). Microvascular density was expressed as the mean number of vessels in these areas. The counts were performed in a masked manner.

EPC analysis To investigate the mobilizing effects of EPC in mice after subconjunctival injected or intraperitoneal injected AMD3100, peripheral blood was collected at 0.5, 1, 6, 24, 96, and 168 hours after the initiation of injection. The viable EPC population in peripheral whole blood was...
analyzed for the expression of Sca-1-PE (eBiosciences) and VEGFR-2-FITC (eBiosciences). Single- and two-color flow cytometric analyses were performed using a FACScan flow cytometer and Cell Quest software (BD Biosciences).

**VEGFR2 expression** The animals were sacrificed at the 7th or the 14th day after alkaliburn of corneal. Sections (4 μm) from formalin-fixed, paraffin-embedded cornea were cut and mounted on slides. Slides were incubated for VEGFR2 (GenWay Biotech Inc., Cambridge, San Diego, CA, dilution: 1/100) and CD34 (CellSciences, Canton, MA, dilution: 1/50) using a biotin-streptavidin-peroxidase detection system (Kit ChemMate, Dako). Diaminobenzidine tetrahydrochloride (DAB) was used for the visualisation of the antibody/enzyme complex. Slides were counterstained with haematoxylin. Negative controls were included in each sample by omitting the primary antibody. Then immunohistochemistry positive index was detected by Medical Image Quantitative Analysis System (MIQAS). Briefly, standard BMP images of file were acquired by digital camera. Distance measurement was corrected with a microscope dedicated micro-scale calibration. After the elimination of violet blue background, blue-negative region and brown-positive region was chosen. Immunohistochemical positive area and optical density were measured for three fields of view of each sample. Immunohistochemistry positive index= Immunohistochemical positive area ×Optical density.

**RESULTS**

**CNV Formation** In order to clarify the therapeutic effects of subconjunctival and intraperitoneal injection of AMD3100 on alkaliburn induced CNV, four groups of experimental animals' inflammatory response and microvessel density were tested. CNV of group A was significantly lower than that of the other three groups. Seven days after alkaliburn, microvessel density of group A was significantly lower than that of group B (Table 1).

<table>
<thead>
<tr>
<th>Injection</th>
<th>0</th>
<th>0.5h</th>
<th>1h</th>
<th>6h</th>
<th>1d</th>
<th>4d</th>
<th>7d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraperitoneal</td>
<td>0.05±0.02</td>
<td>0.11±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.36±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.23±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.12±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Subconjunctival</td>
<td>0.05±0.02</td>
<td>0.07±0.01</td>
<td>0.06±0.02</td>
<td>0.07±0.02</td>
<td>0.06±0.01</td>
<td>0.07±0.01</td>
<td>0.06±0.01</td>
</tr>
</tbody>
</table>

<sup>b</sup>P<0.01 vs subconjunctival injection

**Table 2** EPC number and markers after AMD3100 treatment

**Table 3** EPC number and markers after AMD3100 treatment

**Figure 1** Inflammation index after AMD3100 treatment

**Inflammatory Response** The inflammatory index decreased significantly when group A was compared with the other three groups after 5, 7, 10 and 14 days of AMD3100 treatment (Student-Newman-Keuls, <i>P</i><0.05, Figure 1). On the other hand, the corneal PMN number of group A was significantly less than that of the other three groups after 7 and 14 days of AMD3100 treatment (<i>P</i><0.05, Table 2).

**EPC Mobilization** This study assessed EPC mobilized into the peripheral role of the subconjunctival and intraperitoneal injection of AMD3100 in the C57BL/6 mice. Immediately after intraperitoneal injection of AMD3100 0.5 hour, the number of EPC was significantly increased in peripheral blood (more than 2 fold, from 0.0482±0.02 to 0.1134±0.01). Peak mobilization (up to 7-fold, from 0.0482±0.02 to 0.3558±0.04) of EPC was occurred at 1 day after injection. In contrast, subconjunctival injection of AMD3100, the number of EPC in peripheral blood was not significantly increased (Table 2). IHC positive index of group A was lower than that of the other 3 groups at the 7th and the 14th day, the difference was statistically significant. IHC positive index of group C was higher than that of other three groups at the 7th day and higher than that of group A at the 14th day; the difference was statistically significant (Figure 2).
DISCUSSION

SDF-1/CXCR4 axis not only broadly participates in various inflammatory and angiogenesis-related diseases, but also is related to ocular neovascular diseases. SDF-1/CXCR4 axis was involved in the formation of neovascularization through mechanisms such as attracting inflammatory cells and endothelial cells to inflammatory sites, stimulating the release of matrix metalloproteinase and VEGF, coordinating with VEGF to promote endothelial cells proliferation and migration, preventing apoptosis of neovascular endothelial cells. SDF-1 or CXCR4 knockout mice could suffer with serious gastrointestinal angiodysplasia, cardiac membranous ventricular septal defect and bone marrow disorders. AMD3100 is the SDF-1 receptor-specific antagonists, and do not react with other chemokine receptors. AMD3100 and CXCR4 have a strong binding force, and once combined, the inhibitory effect can be sustained for at least 24 hours. Liles and Broxmeyer found that AMD3100 can mobilize CD34⁺ bone marrow stem cells into the peripheral blood by antagonizing CXCR4 in human and mouse. AMD3100 can improve the mobilization of EPC in bone marrow into the peripheral blood in myocardial infarction patients, so it is possible to use as a new protection treatment for acute myocardial infarction patients. In summary, AMD3100 may have a dual role on the formation of new blood vessels, on the one hand, through promoting the mobilization of EPC from bone marrow into peripheral blood to improve ischemia tissue's blood circulation. On the other hand, AMD3100 can antagonize the biological function of SDF-1, which may inhibit the formation of new blood vessel. We firstly analyzed the therapeutic effects of AMD3100 administrated by subconjunctival and intraperitoneal injection on alkali burn induced CNV in mice. The results showed that compared with the control group at different time points, the subconjunctival injection AMD3100 group's corneal inflammation and vascular density index were significantly lower than other groups, and the number of infiltrated inflammatory cells in corneal tissue was also less. Our experimental results were similar to Grunewald's report. They implanted VEGF release pump in the rat liver to induce the formation of new blood vessels, which have a large number of bone marrow-derived stem cells around. The expression of SDF-1 in the fibroblasts and smooth muscle cells harvested from the new vessels were also significantly increased. However, when AMD3100 pump was implanted into the original site of VEGF pump, the local neovascularization significantly reduced. In contrast, corneal inflammation index and density of new blood vessels of the intraperitoneal injection AMD3100 group were no difference between the control groups, no significant role in inhibition of angiogenesis. We speculate that this may be related to the effects of AMD3100 to mobilize bone marrow EPC into the blood circulation and promote the EPC in the lesions caused by the aggregation. SDF-1/CXCR4 axis is the most important factor, which mediated hematopoietic stem cell homing and mobilization. SDF-1 is mainly in the bone marrow stromal cells and bone marrow endothelial cells. SDF-1 receptor, CXCR4 may be the only CD34⁺ hematopoietic stem/progenitor cells and highly expressed on EPC surface. Yin et al.¹ have shown that SDF-1 can effectively raise the number of EPC and through the promotion of EPC adhesion, proliferation, migration and inhibiting apoptosis induced to improve the function of EPC. Ischemic tissue increased expression of SDF-1 could mediate stem/progenitor cells homing to ischemic tissue involved in the formation of new blood vessels in the transplanted stem cells and EPC on the treatment of ischemic heart disease study. Of our experiments, the whole shooting drug groups of mice using the maximum effective dose of 2.5mg/kg, partial reference to AMD3100 treatment group were given 5ug of EC50 daily subconjunctival injection. The results showed that systemic administration group, the mobilization of EPC have a significant role, and time and dose-response relationship was similar to Yin et al.¹ findings; subconjunctival local administration group, no observed effect on the mobilization of EPC, which may be due to dose less than the minimum effective dose due. Therefore, we speculate that although the corneal alkali burn is an injury-related factor, but it does not significantly stimulate the body to mobilize from bone marrow to peripheral blood EPC, which might be due traumatic stress has not yet reached the threshold value of the mobilization of EPC. Of course, this conclusion requires further study. EPC specific mechanisms involved in the formation of blood vessels remaining in the controversial, some scholars believe that in peripheral blood circulating EPC can be integrated directly with the vascular injury site.
to participate in the formation of new blood vessels. In many animal models of limb ischemia and infusion of cultured human EPC, the 56% of the integration of new blood vessel formation by human EPC. Similarly, in the stent directly in vitro cultivation of EPC can significantly improve the graft re-endothelialization, about 60% of the endothelial cells from the person who planted EPC. Corneal pocket implants microencapsulated particles were induced angiogenesis model assessment EPC in the proportion of new blood vessels and new blood vessels up to 27% of the cells from the blood circulation in the EPC. In addition, sponge-induced granulation tissue, 8% -10% of the vascular endothelial cells are bone marrow-derived cells. These studies have shown that blood circulation can be directly integrated in EPC to neovascularization site and participates in the formation of new blood vessels. Our experiments show that when the whole body to give AMD3100, corneal alkali-burn in mice failed not only to therapeutic angiogenesis, but also to some extent contributed to the formation of new blood vessels; and, in the corneal tissue was also detected in EPC and VEGFR2-specific marker proteins a large number of CD34 expression. These results are consistent with previous studies. Many studies have shown that SDF-1/CXCR4 axis play an important role on the formation of new blood vessels, with multi-faceted promotion of angiogenesis function. Therefore, CXCR4 antagonists theoretically should have a role in inhibiting angiogenesis. The current study suggests that SDF-1 by promoting endothelial cell migration and proliferation, as well as synergy with VEGF and other mechanisms to promote the formation of new blood vessels. For example, Gallagher et al.[9] found that the SDF-1 can reverse the diabetes-induced endothelial progenitor cell homing defect, enhance the expression of SDF-1/CXCR4 in hematopoietic stem cells, promote the migration and proliferation of endothelial progenitor cells. SDF-1 not only through the strengthening of CXCR4 expression in vascular endothelial cells to promote vascular remodeling, but also allows expression of pro-angiogenic role of CXCR4+, VEGFR+ hematopoietic cells in the continuing mobilization of stored state, and thus accelerate the revascularization of ischemic organs. Can be seen, SDF-1/CXCR4 plays an important role in the formation of vascular endothelial cells. Topical application of AMD3100 in treatment of alkali burn induced CNV in mice was possibly by antagonizing CXCR4 SDF-1 binding and thus blocking the biological function of SDF-1. In contrast, systemic application of AMD3100 due to its strong role in the mobilization of EPC, which increased the number of EPC in peripheral blood circulation, promote EPC to the damaged parts of the aggregation of corneal inflammation and thus had a certain degree of promotion of the role of angiogenesis. Our results also show that the formation of new blood vessels and peripheral blood EPC closely related when the corneal alkali burn occurred in mice. So to prevent the aggregation of EPC to the injury of the cornea may be the effective treatment of CNV.

References
7 Yin Y, Huang L, Zhao X. Effects of SDF-1 on number and biological characteristics of bone marrow derived endothelial progenitor cells in mice. Xiangguan Kangli Yiwei Zaishi 2006;15:427–434