·Basic Research·

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

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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 7.57± 1.26 per 0.034mm^2, compared to a number of 14.87± 2.21 per 0.034mm^2 in the control group (P<0.05). On the contrary, the number of CNV in the intraperitoneal injection group was a little higher than that in the control group, 16.34± 1.53 per 0.034mm^2 *vs* 13.26± 1.87 per 0.034mm^2. 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

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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-vessel 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*[1] 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[1].SDF-1 is one member

of the chemokines family, which include four groups, CXC, CX3C, CC, and C (C=cysteine 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[2,3]. Previous studies showed that CXCR4 was central to the retention of HPCs within the bone marrow, and pertu- rbations of the CXCR4:CXCL12 chemotactic interaction within the BM results inmobilization. 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 of filter paper saturated with 1 N NaOH was placed onto the cornea of each one for 10 seconds, followed by rinsing extensively with PBS for 2 minutes. Twenty 8-week-old C57BL/6 mice were randomly divided into four groups (five mice each): group A: subconjunctival injection of 1g/L AMD3100 (5μL/d for a total of 7 injections, Sigma-Aldrich, St. Louis, Mo., USA); group B: subconjunctival injection of balanced salt solution (5μL/d for a total of 7 injections); group C: intraperitoneal injection of AMD3100 (2.5mg/kg per day for a total of 7 injections); group D: intraperitoneal injection of balanced salt solution (0.5mL/d for a total of 7 injections).

Methods

Assessment of inflammatory response and PMN counts Inflammatory response was evaluated by slit lamp. Serial photographs of cornea were taken. The inflammatory index was analyzed as previously described, with some modifications [4]. Briefly, the inflammatory index was analyzed, based on the following parameters: ciliary hyperemia (absent, 0; present but less than 1mm, 1; present between 1 and 2mm, 2; present and more than 2mm, 3); corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present without visible pupil, 3); and intraocular hemorrhage or exudation (absent, 0; present 1). The final inflammatory index result was obtained by summing the crosses of the different parameters divided by a factor of 5. PMN was quantified in the cornea at the 7th and the 14th day after alkali burn. The cornea was fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 5-μm-thick sections and transferred to glass slides. The cornea sections were stained by hematoxylin and eosin staining kit. A cell was deemed a PMN when a multilobed nucleus was clearly identified in cross-section. One cross-sectional slide from the anatomical center of each cornea was analyzed. Five standardized high-powered fields per tissue section were counted using a ×100 objective (two peripheral, two mid-peripheral, and one central). The two peripheral high-powered fields abutted the limbus. The central high-powered fields encompassed the anatomical center of cornea. The two mid-peripheral high-powered fields were midpoint between the central and peripheral high-powered fields. The PMN counts from all five high-powered fields were combined and expressed as PMN counts. The counts were performed in a masked manner.

Microvascular density count The methods of micro-vascular density counting were as described previously. Briefly, corneal microvascular were highlighted by immunostaining with anti-CD34 antigen monolonal 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 (0.034mm^2 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

Table 1 Microvascular density and PMN counts of mice cornea Mean±SD

Group	Microvascular density		PMN counts	
	7d	14d	7d	14d
A	7.6±2.2[b]	11.7±2.0[b]	12.4±3.0[b]	10.0±2.2[b]
B	14.4±2.4	16.2±1.9	21.0±3.4	16.0±2.3
C	13.2±2.4	16.8±1.7	16.2±2.1	15.0±1.6
D	13.6±2.5	17.2±1.9	19.6±2.6	17.4±1.6

[b]P<0.01 *vs* group B,C,D

Table 2 EPC number and markers after AMD3100 treatment Mean±SD

Injection	0	0.5h	1h	6h	1d	4d	7d
intraperitoneal	0.05±0.02	0.11±0.01[b]	0.25±0.04[b]	0.27±0.02[b]	0.36±0.04[b]	0.23±0.04[b]	0.12±0.02[b]
Subconjunctival	0.05±0.02	0.07±0.01	0.06±0.02	0.07±0.02	0.06±0.01	0.07±0.01	0.06±0.01

[b]P<0.01 *vs* subconjunctival injection

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 alkali burn of corneal. Sections (4μm) 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 (Cell Sciences, 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 alkali burn 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 alkali burn, microvessel density of group A was significantly lower than that of group B (Table 1).

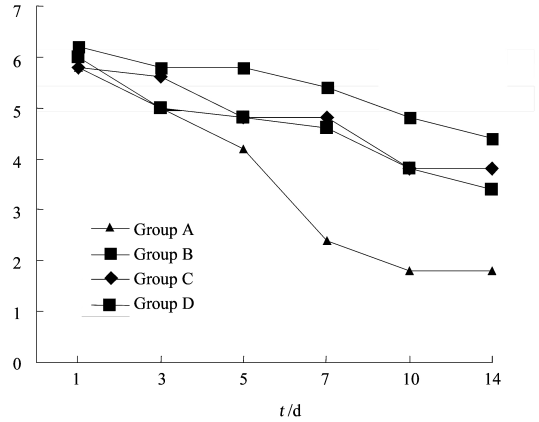

Figure 1 Inflammation index after AMD3100 treatment

Inflammatory Response The inflammatory index decreased significantly when group A was compared with the other three group after 5, 7, 10 and 14 days of AMD3100 treatment (Student-Newman-Keuls, P<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 (P<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).

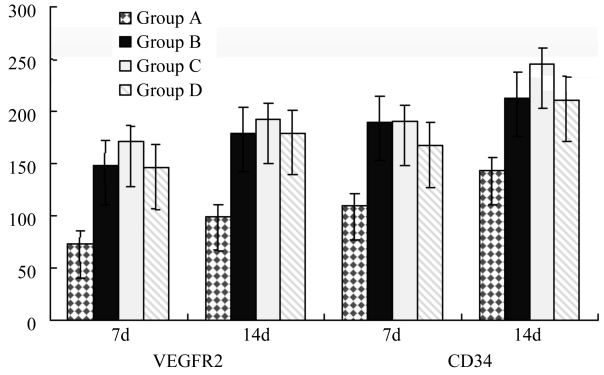

Figure 2 VEGFR2 and CD34 expressions

DISCUSSION

SDF-l/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 [6]. Yin *et al* [7] 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 5μg 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* [8] 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.

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