Exosome-Mediated Delivery of an Anti-Angiogenic Peptide Inhibits Corneal Neovascularization via VDAC1 and Autophagy

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Research Article

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Abstract

Background: Angiogenesis is a crucial reason for the poor prognosis of corneal diseases. KV11, an undecanoic peptide from Kringle Ⅱ domain derived from apolipoprotein(a) is identified to inhibit angiogenesis significantly. Given the poor permeability and instability of small peptide, we aim to use exosome and its anchoring peptide CP05 to deliver KV11 to alkali-burn cornea tissue and inhibit corneal neovascularization.

Methods: In vitro, we tested the effect of EXO-KV11 on inhibition of angiogenesis with the method of tube formation, transwell migration assay and CCK-8. In vivo, we used subconjunctival injection of EXO-KV11 to the alkali-burn cornea-induced corneal neovascularization rat model and fluorescent angiography to directly detect the impact on corneal neovascularization. We also revealed the underlying mechanism by western blot.

Results: EXO-KV11 was verified to suppress endothelial cells tube formation, migration and proliferation in vitro by tube formation assay, transwell migration assay and CCK-8 assay. EXO-KV11 was more efficiently delivered to cornea tissue and suppressed more corneal neovascularization in alkali burn-induced corneal neovascularization model. Furthermore, VDAC1 might be potential receptor for KV11 and KV11 stimulated PERK and blocked up autophagy flux.

Conclusions: We used exosomes to delivery KV11 into cells and cornea tissue. We reported that EXO-KV11 could inhibit angiogenesis more than KV11 both in vitro and in vivo. Thus exosome-CP05 drug delivery system has promising prospect in the future.

Introduction

As a critical medium of refraction, normal corneal structure is indispensable. One of the main reasons for corneal optical clarity is avascularity.[1, 2] However, under the condition of hypoxia, inflammation, trauma, and other harm, neovascularization is facilitated to grow into cornea from corneal limbal vascular net and have ill effects on the opacity of cornea, which names corneal neovascularization (CoNV), even leading to blindness.[3, 4] At present, potential therapies range from pharmacologic agents-either broadly blocking inflammation (e.g. steroids) to targeted molecular therapies (e.g. anti-VEGF agents)-to surgical vessel ablation and even gene therapies. [5] However there are lots of drawbacks and limitations as for existing therapies. Although steroids have been the mainstay of CoNV treatment for decades, notorious for multiple side-effects, including steroid-induced glaucoma, acceleration of cataract formation and increased infection risk.[1] And anti-VEGF agents are less effective in the presence of mature CoNV. [6] Photodynamic therapy (PDT) reduces mature vessels, but it's destructive and carries the risk of collateral damage to the surrounding cornea and limbal stem cells. Mainstay treatments of CoNV have partial efficacy and associated side effects, revealing the need for novel treatments.

Kringle domain exists extensively in plasminogen and apolipoprotein, verified to play a key role in the inhibition of angiogenesis ,especially Kringle Ⅱ(KⅡ).[7–11] Plasminogen Kringle Ⅱ is confirmed to suppress

angiogenesis in gastric cancer, hepatocellular carcinoma and corneal neovascularization.[12–14] And Don J. Davidson et al. found that small peptide consisting of YTTNPRKLYDY derived from plasminogen K α could inhibit endothelial cells migrating better.[15] Undecanoic peptide YTMNPRKLFDY derived from apolipoprotein(a) could significantly inhibit retinal neovascularization. [16, 17] However, as is well-known, small peptides as a sort of drugs take the disadvantages of instability and membrane impermeability and so on. [18–20] Therefore, we need a kind of drug carrier to prolong the half-life and accelerate the drug to enter the cells.

More and more attention is paid to Exosome, a sort of extracellular vesicles, especially on the aspect of drug delivery and target treatment. [21–24] Exosome is a kind of 30-150nm extracellular vesicle, secreted by almost all kinds of cells.[25–28] Recent years, it has been realized that exosome has the function of intercellular communication, which means exosome could directly deliver drugs into cells as a drug microcarrier, increasing the effect of drug and the period of circulation, enhancing drugs stability and prolonging half-life period.[29, 30] Xianjun Gao et al illustrate that an anchoring peptide which names CP05 could target exosome membrane marker protein CD63 thus loading kinds of sources of drugs.[24, 31] Xue Dong et al. loaded KV11 on exosomes by using CP05 and demonstrates this system has the potential to treat retinal neovascularization in a highly effective but less invasive way.[32]

However, the mechanism about why EXO-KV11 is more effective than KV11 alone and whether EXO-KV11 has the same effect on corneal neovascularization are not clear. Thus, we performed further experiments to elucidate the reason about gaps between EXO-KV11 and KV11 and the effect on CoNV.

Results

KV11 binds to Exosomes efficiently through anchoring peptide CP05

Exosome was identified by TEM as 30-150nm disc-shaped vesicles (Figure 1A). NTA showed that the average diameter was about 127nm and the concentration was $2.15 \times 10^9 \pm 6.55 \times 10^7$ particles/ml (Figure 1B) and exosome marker protein CD63 was identified positive by western blotting (Figure 1C).

To evaluate the binding rate, we performed Apogee flow system for EXO-KV11. The results were as follows: when the concentration ratio of EXO:KV11 was 1:4 (6.25ug/ml:25ug/ml), the binding efficiency was 87.5%(Figure1D). As the results showed, the 1:4 concentration ratio of EXO:KV11 was the best, thus we used the ratio to continue following experiments.

EXO-KV11 inhibits VEGF-induced endothelial cell proliferation, migration, and tube formation in vitro

Formation of vascular networks requires endothelial cell (EC) tube formation. Therefore, we conducted the tube formation assay. Both EXO-KV11 and KV11 pretreatment showed dramatic inhibitory effect on EC tube formation ($p < 0.001$) and more significant than Avastin ($p < 0.001$), besides EXO-KV11 had more

obvious effect than KV11 ($p<0.05$) (Figure 2A). We also analyzed the effect on EC migration and EXO-KV11 could also inhibit VEGF-stimulated EC migration prominently ($p<0.01$), whereas KV11 had no intergroup difference contrast to control group ($p>0.05$) (Figure 2B). Furthermore, we performed CCK-8 assay and the results showed both EXO-KV11 and KV11 suppressed the EC proliferation remarkably ($p<0.01$) (Figure 2C).

EXO-KV11 upregulates the expression VDAC1 in vitro

Voltage-dependent anion channel 1 (VDAC1) is proposed to be receptor for plasminogen Kringle 5 on human endothelial cells. [33] Hence, we speculated that KV11 derived from apolipoprotein(a) could be the potential ligand for VDAC1 and focused on the expression of VDAC1. EXO-KV11 and KV11 pretreatment for 24h enhanced the expression of VDAC1 significantly ($p<0.001$) (Figure 3A, C). We performed further experiments and shortened pretreatment time to 12 h. The results showed EXO-KV11 still upregulated the expression of VDAC1 ($p<0.05$) but KV11 did not ($p>0.05$) (Figure 3A, C), implying exosome-loaded KV11 could promote VDAC1 one step ahead.

HUVECs were pretreated with EXO-KV11, KV11 or EXO for 12h or 24h and then stimulated with 20ng/mL VEGF for 30 min. Representative western blots for changes in the expression of VDAC1 (12 h or 24 h) (A). Avastin was used as a positive control and normal saline was used a negative control. 30 μ g total protein from each sample was loaded.. n=3-6 independent experiments. The data represent as mean $\pm$ SEM.* $p<0.05$, ** $p<0.01$, *** $p<0.001$, one way ANOVA followed by LSD (Least significance difference test) in (B, C).

EXO-KV11 suppresses autophagy flux and Claudin-5 expression in vitro

In view that VDAC1 is relative with endoplasmic reticulum (ER) stress, we tested the expression of PERK. We observed EXO-KV11 and KV11 induced the enhancement of PERK and EXO-KV11 promoted a lot more ($p<0.001$) (Figure 4A, B). We also investigated autophagy flux and the results demonstrated that the expression of LC3B β and LC3B α were elevated simultaneously ($p>0.05$) (Figure 4C, D, E). Additionally, the adaptor protein p62/SQSTM1 was verified to be upregulated and EXO-KV11 enhanced p62 expression more predominantly than KV11 alone ($p<0.001$) (Figure 4D, E). EXO-KV11 and KV11 stimulated expression of cleaved caspase-3 ($p<0.01$) (Figure 4F, G).

We also tested the expression of Claudin-5, the tight junction of ECs, relevant with vascular permeability and the results showed KV11 upregulated the expression of Claudin-5 (Figure 4H, I, J). On the contrary, EXO-KV11 reduced the expression of Claudin-5 (Figure 4H, I, J).

EXO-KV11 inhibits corneal angiogenesis in vivo

We determined whether KV11 could inhibit corneal neovascularization in an animal model of alkali burn injury. Rats received subconjunctival injections of normal saline, KV11 and EXO-KV11 containing an equivalent amount of KV11. 14 days after alkali-burn, the corneas treated with normal saline exhibited

excessive ingrowth of neovessels from the limbus into the central corneal stroma, and those treated with KV11 decreased new blood vessel formation in the corneas moderately at 14 d ($p < 0.05$) (Figure 5A, B). By contrast, a subconjunctival injection of EXO-KV11 exerted its function at 7d whereas KV11 alone not and EXO-KV11 substantially decreased new blood vessel formation in the corneas at 14 d ($p < 0.001$) (Figure 5A, B). H&E staining and CD31 IHC staining were coincident with the above (Figure 5C). For an additional quantitative analysis of anti-angiogenesis effect, the extent of corneal neovascularization was assessed by perfusing rats with FITC-dextran and detecting the area ratio in the cornea. The analysis showed a significant reduction in the relative fluorescence area of EXO-KV11-injected corneas compared with those of KV11- or normal saline-injected controls (Figure 5D, E). These data suggested that EXO-KV11 exerted a substantially greater inhibitory effect on corneal neovascularization than KV11 alone did..

EXO-KV11 induces the upregulation of VDAC1/PERK/cleaved-caspase-3

Rats were sacrificed after 14 d and corneas were extracted. We performed western blotting to test the expression of VDAC1 and PERK which were verified to be enhanced in vitro. The results presented that KV11 didn't alter the expression of VDAC1 and PERK as it did in vitro ($p > 0.05$) (Figure 6A, B, D, E). Astonishingly, KV11 downregulated the expression of Claudin-5 ($p < 0.01$) (Figure 6A, C). KV11 induced a moderate increase in cleaved-caspase-3 ($p < 0.001$) (Figure 6G, H) and had no impact on LC3B β /LC3B α ($p > 0.05$) (Figure 6D, F).

On the other hand, EXO-KV11 could augment the expression of VDAC1 ($p < 0.001$) and PERK ($p < 0.01$) (Figure 6A, B, D, E). There was also no difference at the aspect of the ratio of LC3B β to LC3B α treated with EXO-KV11 ($p > 0.05$) (Figure 6D, F). Additionally, EXO-KV11 increased cleaved-caspase-3 more than KV11 alone ($p < 0.001$) (Figure 6G, H). EXO-KV11 still reduced Claudin-5 expression in vivo ($p < 0.01$) (Figure 6A, C)

Discussion

Apolipoprotein(a) [apo(a)] contains numerous kringle domains and apo(a) kringle V shows significant sequence homology with plasminogen kringle 5, which is reported to be a potent angiogenesis inhibitor. [12, 34] Apo(a) kringle 5 is identified to inhibit tube formation and angiogenesis.[35, 36] KV11, a small peptide from apo(a) kringle 5 is also indicated to inhibit retinal neovascularization.[16, 17, 37] And our work illustrates that KV11 inhibits CoNV remarkably and EXO-KV11 behaves better.

Voltage-dependent anion channel 1 (VDAC1), surface-expressed glucose-regulated protein 78 (GRP78) and human laminin $\alpha 3$ chain G1 domain (LG1) are defined as the receptors for plasminogen Kringle V.[15, 34, 38, 39] At this stage, it is not known if apo(a) KV11 interacts with any endothelial cell-surface protein. Further work to identify the KV11 peptide receptor would help reveal the molecular mechanism of KV11 mediated anti-angiogenic function. Our work here demonstrates that KV11 upregulates the expression of VDAC1 after pretreatment for 24 h in vitro and ultimately enhance cleaved caspase-3, which means KV11 might be a potential ligand for VDAC1 and induce apoptosis in endothelial cells.

Given VDAC1 also participates in endoplasmic reticulum (ER)-mitochondria cross-talk, and in the regulation of autophagy, [40] we conduct experiments to illuminate whether KV11 has impact on ER stress and autophagy via VDAC1 or not. Our work shows KV11 stimulates PERK, which is one of three ER-transmembrane sensors. [41, 42] Nevertheless, there is no intergroups difference at the aspect of LC3B β /LC3B α while KV11 increases the expression of p62. As is known, when autophagy flux is inhibited, the p62 levels increase.[43] Thus KV11 promotes ER stress and blocks up autophagy flux. Autophagy serves as a pro-survival mechanism under ER stress conditions[44], and accumulation of misfolded protein and inhibition of digestion by autophagy might lead to EC homeostasis disorder and apoptosis. Additionally, this mechanism is not caused via VDAC1 because we detect the change after pretreatment for 12 h while the upregulation of VDAC1 is not detected at that time.

Claudin-5, a significant component of tight junction transmembrane protein in vasculature,[45] only exists in the stroma of human cornea.[46, 47] We disclose that KV11 increases Claudin-5 expression in HUVECs, meaning KV11 strengthens tight junction and suppresses vascular permeability.

In view of high-efficiency and stability, there is more and more attention paid to exosome as a drug carrier and scientists devote themselves to making exosome load larger amount and more kinds of drugs, even targeted delivery by modified surface receptors.[25, 48] Anchoring peptide CP05 is the outstanding one among all. CP05 functions through binding with CD63, a common marker protein of exosomes.[24] We make exosomes deliver KV11 to HUVEC through the fusion of CP05 and KV11. Our results suggest that EXO-KV11 suppresses angiogenesis better than KV11 alone.

In vitro, EXO-KV11 inhibits HUVEC tube formation and migration more than KV11 alone, although they equal at the aspect of inhibiting EC proliferation. Furthermore, our work exhibits EXO-KV11 enhances the expression of VDAC1 earlier than KV11 alone (EXO-KV11 promotes VDAC1 after pretreatment for 12h while KV11 does not, although both EXO-KV11 and KV11 upregulates VDAC1 expression after pretreatment for 24 h). We also reveal that EXO-KV11 increases the expression of PERK and p62 more than KV11. In vivo, the results of fluorescent angiography and anterior segment photography present EXO-KV11 suppresses CoNV more than KV11. EXO-KV11 upregulated the expression of VDAC1 and PERK in the cornea tissue while KV11 is not, which might induce that EXO-KV11 activates more caspase-3 than KV11 alone. Astonishingly EXO-KV11 and KV11 have distinguished effect on Claudin-5, although KV11 increases Claudin-5 expression in vitro, it reduces Claudin-5 expression in vivo, which is the same as the effect of EXO-KV11 in vitro and in vivo. The underlying mechanism of KV11 effect on Claudin-5 needs further study.

Exosome is superior in stabilizing the status of drugs, prolonging the half-life. Besides, our experiments indicate it could make KV11 function more quickly and significantly in vitro and in vivo. We prudently speculate EXO-KV11 might compensate the drawbacks of KV11 alone in poor permeability and instability and function sufficiently. However, the precise mechanism of discrepancies between EXO-KV11 and KV11 needs more exploration. It's also worth noting that exosome derives from all sorts of culture medium and fluid which means the content of exosomes might not be the same and have uncertain effect on the

same experiments. After all, though it seems like a promising prospect, exosomes still have a long way to go as a mature drug delivery vehicle.

Conclusion

In summary, we demonstrate that EXO-KV11 could inhibit angiogenesis better than KV11 with the method of tube formation, transwell migration and CCK-8 assay in vitro and alkali-burn-induced corneal neovascularization model in vivo. We also indicates EXO-KV11 promotes VDAC1 and PERK and blocks up autophagy flux more than KV11 alone, implying exosome-loaded peptide has enormous advantages.

Methods And Materials

Cell culture.

Human umbilical vein endothelial cells (HUVECs) were purchased from Zhejiang Meisen Cell Technology (Zhejiang, China). HUVECs were used in passage 3-6. The cells were cultured in ECM (ScienCell, US) in a 37°C incubator with humidified atmosphere of 5%CO₂/95% air. Cells were digested to conventional passage or cryopreservation when the cells grew to 70% confluence.

Preparation and identification of Exosomes from HUVEC culture medium.

The FBS was ultracentrifuged at 100,000g for 16 h to deplete FBS-derived exosomes before experiments. We isolated HUVEC-derived exosomes by total Exosome isolation kit (Thermo Scientific US). The HUVECs were cultured in T75 for 48-72 h and then the culture medium was collected and centrifuged at 2000g for 30min to remove cells and debris. The supernatant was subjected to incubation with 1/2 volume of total exosome isolation kit (Thermo Scientific, US) overnight at 4°C as the kit introduction illustrates. Then the mixture was centrifuged at 10000g for 1 h at 4°C to pellet HUVEC-exosomes. The pelleted exosomes that each milliliter of supernatant generated were resuspended with 10ul normal saline and aliquoted for nanoparticle tracking analysis (Malvern, Nanosight NS300, UK), transmission electron microscopy (TEM, Leica JEM-1400, Germany) and western blot. The protein concentration of extraction was about 2ug/ul by BCA (Beyotime Biotechnology, China).

Preparation and identification of EXO-KV11

The KV11 sequence was YTMNPRKLFDY, the KV11(CP05) sequence was YTMNPRKLFDYCRHSQMTVSRL. KV11 and KV11(CP05) were synthesized as a single fusion peptide via peptide bond without space by ChinaPeptides (Shanghai, CHINA) with 98% of purity.

We aimed to combining Exosomes with FITC-labeled (CP05) KV11 in order to evaluate the binding rate. For this, (CP05)KV11 was incubated with exosomes overnight at 4°C. Analyses of labeled samples were performed on an Apogee A-50 Micro flow cytometer (FC) (Apogee Flow Systems, UK) equipped with 488-nm(green) that were dedicated to KV11(FITC) detection. Flow cytometry performance was first verified on

each day of measurement using a reference bead mix (ApogeeMix, Apogee Flow Systems composed of a mixture of non-fluorescent silica beads with diameters of 180 nm, 240 nm, 300 nm, 590 nm, 880 nm, and 1300 nm with a refractive index (RI) of 1.42 and 110-nm and 500-nm green fluorescent (excited using a blue laser) latex spheres with an RI of 1.59. Acquisition was performed in duplicate at a sample flow rate 0.75 μ l/min and a 150- μ l sample volume for 180 s.

The exosome concentration (number of Exosomes per μ l of prepared purified MVs) was measured automatically based on the sample volume, cytometer flow rate and the number of fluorescence-positive events(n). Exosomes were gated using medium angle light scatter (MALS) and fluorescence triggering channel and defined as KV11(FITC)-positive events as observed in color fluorescence plots .

CCK-8 assay

The CCK-8 (Cell Counting Kit-8, Dojindo, Japan) assay was utilized to test the influence of EXO-KV11 on HUVECs proliferation ability contrast to KV11. HUVECs were cultured as set forth, and were starved for 24h in FBS-free ECM, and each group respectively were subjected to incubation with EXO-KV11(6.25 μ g/ml+25 μ g/ml),KV1125 μ g/ml, EXO6.25 μ g/ml, normal saline of the same volume or Avastin10 μ g/ml. After 24h, cell suspension of every group was prepared and diluted to the concentration of 5×10^4 cells/ml. 100 μ l diluted cell suspension of each group was seeded into 96-well plates. Each well contained 20ng/ml VEGF (hVEGF165, Proteintech, China) ECM and was treated separately with EXO-KV11(6.25 μ g/ml+25 μ g/ml),KV1125 μ g/ml, EXO6.25 μ g/ml,normal saline of the same volume or Avastin10 μ g/ml, Roche, Switzerland. Then HUVECs were cultured for 24h. After that the CCK-8 solution (10 μ l) was added to each well and incubated for 2h at 37°C. The optical density (OD) was read at 450 nm on a microplate reader (Molecular Devices, SpectraMax 190, China). Cells from 3-5 wells were counted at each time point, and the experiment was repeated 3 times.

Tube formation assay

We evaluated the ability of tube formation contrast by utilizing the capacity of endothelial cells formatting tube on Matrigel (Corning, US). HUVEC was cultured as set previously mentioned. Before the experiment, we put the Matrigel at 4°C overnight and precooled the pipette and 96-well plates. 60 μ l Matrigel was put onto the 96-well plate on ice without air bubbles and then put into 37°C cell incubator for 30min to be solidified. HUVEC was digested to cell supernatant and diluted to 15×10^4 cells/ml. 100 μ l diluted cell suspension of each group was put into Matrigel-covered 96-well plates. Each well was treated separately with EXO-KV11(6.25 μ g/ml+25 μ g/ml),KV1125 μ g/ml, EXO6.25 μ g/ml,normal saline of the same volume or Avastin10 μ g/ml under the stimulation of 20ng/ml VEGF. Each group had 3-5 replicates and then continued to culture for 6h. Photographs were taken under microscope (Olympus BX41, Japan). We utilized software image j to analyze the amount of meshes to evaluate the capacity of HUVEC tube formation in vitro.

Transwell migration assay

Transwell (Corning, US) consisted of upper and lower chambers separated by microporous polycarbonate membranes. We put HUVEC in the upper chamber containing our treatment and put ECM containing 20ng/ml VEGF in the lower chamber to test the ability of migration. HUVEC was cultured as set previously mentioned, and then HUVEC was digested to cell supernatant and diluted to 15×10^4 cells/ml. we added 600ul ECM containing 20ng/ml VEGF in the lower chamber and 100ul cell supernatant in the upper chamber with EXO-KV11(6.25μg/ml+25μg/ml),KV11 25μg/ml, EXO 6.25μg/ml,normal saline of the same volume or Avastin 10μg/ml in each group. And the plates were set in the cell incubator for 24h. Then we pipetted out the fluid in the upper chamber and wipe the cells on the upper chamber surface softly by wet cotton swab. The chamber was fixed by 4% paraformaldehyde for 30 min at room temperature. Cells were stained with crystal violet (Beyotime, China) and photographed under microscope. The results were analyzed by software image j.

Western blot

The protein of ECs and cornea tissue was extracted with RIPA lysis buffer (Thermo Scientific, USA) with protease inhibitor. Protein lysates were electrophoresed through SDS-PAGE gels and transferred onto NC membranes (Millipore, Germany). The membranes were blocked with TBS blocking buffer (LI-COR, US) for 1.5 h and incubated with primary antibodies at 4°C overnight. Target proteins were detected by specific primary antibodies including CD63 (cat.no. 25682, 1:1000, Proteintech, China), VDAC1 (cat.no.55259, 1:1000, Proteintech, China), PERK(C33E10) (cat.no.3192, 1:1000, Cell Signaling Technology, US), SQSTM1/p62 (cat.no.5114, 1:1000, Cell Signaling Technology, US), LC3B (cat.no.ab192890, 1:1000, Abcam), caspase-3 (cat.no.19677, 1:1000, Proteintech, China), claudin-5 (cat.no.bs-10296R, 1:500, Bioss Antibodies, China) or β-actin (cat.no.66009, 1:1000, Proteintech, China)-were used as loading control.

The bound primary antibody was detected by mouse anti-rabbit IgG-CFL 790 (cat.no.sc-516253 1:200, Santa Cruz, US) and m-IgGκBP-CFL 790 (cat.no.sc-516181 1:1000, Santa Cruz, US) respectively. The Odyssey CLx western blotting analysis system (LI-COR, US) was applied. Quantification of WB signal was done by Image J software. Protein expression values were divided by their β-actin respectively. Each experiment was performed at least three times.

Rat model of alkali burn injury-induced corneal neovascularization

100 Sprague Dawley (SD) rats with a mean weight of 200 g (Jiangsu Huafukang Biotechnology, China) were used in the study. All animal studies were approved by the Peking People's Hospital Ethics Committee (No.20210019) and in accordance with the regulations on the management of laboratory animals (the national science and technology commission).

Rats were anesthetized by exposure to 2.5% isoflurane. The center of the right cornea was cauterized by pressing a filter paper (3 mm, soaked in 1 mol/L NaOH solution and strained off any excess liquid) for 30 s (day 0). After that, 15 mL normal saline was used to fully clean the NaOH solution. Each injured rat eye

received a subconjunctival injection of 100μL of EXO-KV11(25μg), KV11(25μg) and normal saline every other day. The cauterized corneas were monitored under slit-lamp biomicroscopy (TOPCON, 75-1, Hasunnuma-cho, Itabashi-ku, Tokyo, Japan) on day 1,4,7,14.

Analyses of corneal neovascularization

The area of corneal neovascularization was determined as previously described.[49] Each photograph was analyzed at the same magnification using Image J software. Through the use of a reticule, the vessel length(L) from the limbus and the number of clock hours (C) of the cornea involved were measured, and the vascularized area= $C/12 \times 3.1416[r^2 - (r-L)^2]$, where r=3 mm and is the radius of the rat cornea. Investigators who performed this assay were not blinded to the treatment.

Furthermore, the area of corneal neovascularization was also analyzed by intracardial injection performed as previously described[50] and the protocol was modified moderately. In brief, rats were anesthetized by ketamine (80mg/kg)/xylazine(10mg/kg). And then they were perfused with 25mg/ml fluorescein isothiocyanate (FITC)-labeled dextran (MW 2000,000, Sigma). After 1 min, the eyes were enucleated and fixed in paraformaldehyde solution (4.0%) for 30 min. After the posterior segment of the eyeball was removed, the anterior segment was mounted on a glass slide and observed using a fluorescence microscope (Olympus AX80, Japan). Corneal neovascularization was quantitatively evaluated by detecting the ratio of the fluorescent area of neovascularization to the area of total cornea using the image pro plus software.

H&E staining and immunostaining of corneal neovascularization

Corneal were carefully dissected under microscopy and rinsed in normal saline. Paraformaldehyde-fixed corneas were embedded in paraffin and sectioned at 4μm for histological and immunohistological analysis. For the visualization of three layers of cornea structure, three non-consecutive sections were stained with hematoxylin and eosin (H&E) in each cornea.

To directly observe CoNV, section were incubated with primary antibodies to rat CD31 (1:500, ab281583, Abcam, US) at 4°C overnight. This was followed by rinse 3 times in PBS. The staining was visualized through the use of horseradish peroxidase (HRP) detection system (Servicebio, China), followed by counterstaining with hematoxylin.

Statistical analysis

All data were expressed as mean±standard deviation(SD) for the respective groups. Statistical analyses were performed with GraphPad Prism software. The Student's t test, two-way ANOVA followed by a Least Significant Distance (LSD) post hoc test were used by SPSS 23.0 statistical software for analyzing the data. For all measurements, a p < 0.05 was considered statistically significant. The following designations for the p value were used in the manuscript figures: ns p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001.

Abbreviations

CoNV
Corneal neovascularization
PDT
Photodynamic therapy
EC
Endothelial cell
VDAC1
Voltage-dependent anion channel 1
ER stress
Endoplasmic reticulum stress:LSD:Least significance different test
apo(a)
Apolipoprotein (a)
EXO
exosome
KV
Kringle 5
EXO-KV11
Exosome-loaded KV11
GRP78
Glucose-regulated protein 78
LG1
Laminin α 3 chain G1 domain
HUVECs
Human umbilical vein endothelial cells
TEM
Transmission electron microscopy
FC
Flow cytometer
FITC
Fluorescein isothiocyanate
RI
Refractive index
MVs
Microvesicles, MALS:Medium angle light scatter
CCK-8
Cell Counting Kit-8
OD
Optical density

SDS–PAGE

Sulfate–polyacrylamide gel electrophoresis

SD rats

Sprague Dawley rats

NC membrane

Nitrocellulose filter membrane

L

Length

C

The number of clock hours

H&E

Hematoxylin and eosin

HRP

Horseradish peroxidase

SD

Standard deviation

Declarations

Ethic approval and consent to participate

All animal experiments were approved by Peking People's Hospital Ethics Committee (No.20210019) and in accordance with the regulations on the management of laboratory animals (the national science and technology commission).

Consent for publication

All authors agreed to submit this manuscript.

Availability of data and materials

All data included in this study are available upon request by contact with the corresponding author.

Competing interests

To the best of our knowledge, the authors have no conflicts of interest, financial or otherwise.

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Author's contributions

Conceptualization: Yu WZ, Chen WQ and Du W; Methodology: Chen WQ, Yu WZ and Du W; investigation: Chen WQ and Du W; Writing—original draft: Chen WQ; Writing—Review & Editing: Du W, Chen WQ and Yu WZ; Supervision: Yu WZ and Du W. All authors read and approved the final manuscript.

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Figures

Figure 1

preparation of exosome and binding efficacy of exosome and KV11(CP05)

(A) Representative transmission electron microscopy image of HUVEC-derived EXOs. (B): Nanosight analysis of the size distribution of EXOs derived from HUVECs. (C): Western blot analysis for the expression of CD63 proteins in HUVEC

(B) s-derived exosomes. 20μg total protein from exosomes. (D): EXOs were incubated with FITC-labeled KV11(CP05) of different concentrations overnight at 4°C. After incubation, the percentage of FITC-positive events is indicated in each histogram.

Figure 2

EXO-KV11 suppresses VEGF-induced angiogenic effects

(A): HUVECs were treated with EXO-KV11, KV11, EXO or Avastin for 24h. After incubation tube formation ability of HUVECs cultivated for 6h with the stimulation of VEGF and EXO_{KV11}, KV11, EXO or Avastin. (B): HUVECs were treated with EXO-KV11, KV11, EXO or Avastin for 24h. After incubation transwell migration assay of HUVECs cultivated for 24h with upper chamber containing EXO_{KV11}, KV11, EXO or Avastin and lower chamber containing VEGF. (C) HUVECs were treated with EXO-KV11, KV11, EXO or Avastin for 24h. CCK-8 assays of HUVECs cultivated for 24h with VEGF stimulation and OD was read.

Figure 3

Effect of EXO-KV11 on VDAC1 expression in vitro

HUVECs were pretreated with EXO-KV11, KV11 or EXO for 12h or 24h and then stimulated with 20ng/mL VEGF for 30 min. Representative western blots for changes in the expression of VDAC1 (12 h or 24 h) (A). Avastin was used as a positive control and normal saline was used a negative control. 30μg total protein from each sample was loaded.. n=3-6 independent experiments. The data represent as mean±SEM.*p<0.05, **p<0.01, ***p<0.001, one way ANOVA followed by LSD (Least significance difference test) in (B, C).

Figure 4

Effect of EXO-KV11 on PERK, p62, LC3B/LC3B, cleaved caspase-3 and Claudin-5 expression in vitro

HUVECs were pretreated with EXO-KV11, KV11 or EXO for 12h or 24h and then stimulated with 20ng/mL VEGF for 30 min. Representative western blots for changes in the expression of PERK, p62, LC3B/LC3B, cleaved caspase-3 and Claudin-5 (A, C, F, H). Avastin was used as a positive control and normal saline

was used a negative control. 30μg total protein from each sample was loaded. n=3-6 independent experiments. The data represent as mean±SEM.*p<0.05, **p<0.01, ***p<0.001, one way ANOVA followed by LSD (Least significance difference test) in (B, D, E, G, I).

Figure 5

Effect of EXO-KV11 on corneal neovascularization in the alkali-burned corneas.

Representative gross images of eyes treated with EXO-KV11, KV11 or control (normal saline) at 1, 4, 7, 14 d after injury (A). The data of CoNV area represent in (B). Representative H&E and CD31 staining images treated with EXO-KV11, KV11 or control (normal saline) at 14 d, scale bar: 25 μ m. (C) Representative images with FITC-dextran in corneas from alkali-burn rats treated with EXO-KV11, KV11 or normal saline after 14 d.(D) The data of the ratio of CoNV area to cornea area represent in (E). n=6-8 independent experiments. The data represent as mean $\pm$ SEM.*p<0.05, **p<0.01, ***p<0.001, one way ANOVA followed by LSD (Least significance difference test).

Figure 6

Effect of EXO-KV11 on VDAC1, PERK, LC3B α /LC3B β , Claudin-5 and cleaved caspase-3 expression after corneal alkali burns.

Representative western blots for changes in the expression of VDAC1, PERK, LC3B α /LC3B β , Claudin-5 and cleaved caspase-3. 30 μ g total protein from each sample was loaded. Quantification of blots shown in (A, D, G). n=3-6 independent experiments. The data represent as mean $\pm$ SEM.*p<0.05, **p<0.01, ***p<0.001, one way ANOVA followed by LSD (Least significance difference test) in (B, C, E, F, H).

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