Suppressive effects of selectin inhibitor SKK-60060 on the leucocyte infiltration during endotoxin induced uveitis

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Background: It is well known that selectin is involved in the development of endotoxin induced uveitis (EIU), and has a major role in leucocyte infiltration. Recently, a novel selectin inhibitor (SKK-60060) that can block P and L selectins in vitro has been developed. This study was designed to investigate the anti-inflammatory effects of SKK-60060 on the inflammatory reaction during EIU in rats by studying leucocyte-endothelium interactions.

Methods: EIU was induced in Lewis rats by footpad injection of lipopolysaccharide (LPS). SKK-60060 was administered 15 minutes before LPS injection, and its suppressive effects on inflammatory leucocyte behaviour were evaluated in vivo with acridine orange digital fluorography; the diameters of retinal arteries and veins were also measured. After these studies, aqueous humour was collected to evaluate leucocyte infiltration and protein leakage.

Results: After LPS injection, rolling leucocytes were observed in major retinal veins, followed by leucocyte infiltration into the vitreous cavity. Following treatment with SKK-60060, leucocyte rolling was significantly inhibited in the retinal veins ($p < 0.01$), and subsequent leucocyte infiltration into the vitreous cavity was also significantly suppressed ($p < 0.01$). Retinal vasodilation was also substantially suppressed in SKK-60060 treated rats ($p < 0.01$). Similarly, leucocyte infiltration and protein leakage into the aqueous humour were reduced significantly by SKK-60060 ($p < 0.01$).

Conclusions: SKK-60060 treatment significantly inhibited the inflammatory reaction induced by LPS. Its inhibitory effects on P and L-selectin resulted in suppression of leucocyte infiltration and the subsequent inflammatory reaction caused by accumulated leucocytes. The current findings suggest that SKK-60060 may be useful in the management of uveitis.

Endotoxin induced uveitis (EIU) is an animal model of ocular inflammation induced by subcutaneous injection of lipopolysaccharide (LPS).^{1,2} It is characterised by blood-ocular barrier disruption and leucocyte infiltration. Recent studies have shown the contribution of adhesion molecules in the leucocyte infiltration during EIU.^{2–10} It is generally believed that leucocyte infiltration occurs through a multistep cascade of events involving sequential leucocyte rolling along vessel walls, firm adhesion to endothelium, and emigration out of the blood stream into inflamed tissues.^{11,12} We have demonstrated that the cell adhesion molecule P-selectin mediates the step of leucocyte rolling in the retinal veins of rats during EIU, which results in the leucocyte infiltration into the vitreous cavity.¹⁰ P-selectin is a member of the selectin family of adhesion molecules that includes other selectins: E-selectin and L-selectin. It is thought that L-selectin has two properties in the multistep cascades of leucocyte infiltration. Firstly, L-selectin mediates the early phase of leucocyte rolling.^{11–13} Secondly, it transduces the signal to the leucocyte that activates the adhesion and emigration steps.^{14,15}

Although all three selectins have been thought to participate in the process of leucocyte infiltration, Robinson *et al*¹⁶ have shown that P and L-selectin are important in regulating leucocyte behaviour in inflammation. Thus, it would be worthwhile to synthesise an inhibitor against both P-selectin and L-selectin. Sulfatide, sulfate galactosylceramide, has been found to be a good ligand for these selectins,^{17–19} with protective effects against selectin dependent inflammatory responses.^{20,21} However, this substance is difficult to dissolve, and therefore has limited potential as a medicine. Recently, a novel selectin inhibitor (SKK-60060) has been developed that is similar to the natural sulfatide. SKK-60060 is 2-(tetradecyl)hexadecyl O-(3-O-sulfo- α -L-fucopyranosyl)-

(1 $\rightarrow$ 2)-3-O-sulfo- β -D-galactopyranoside disodium salt. The activity in vitro was measured in adhesion assays as the inhibition of the binding of HL-60 cells (sLe^x expressing) to recombinant human P, L, and E-selectin-IgG fusion proteins on plates. The results demonstrated that SKK-60060 was able to inhibit HL-60 cells binding to the selectin fusion proteins with greater potency than the sLe^x tetrasaccharide itself, 76% and 69% blockage of adhesion being observed with P-selectin and L-selectin, respectively.²² It is readily soluble and recently has been demonstrated to have protective effects against retinal ischaemia reperfusion injury in rats.²³

Since SKK-60060 inhibits P-selectin and L-selectin, it should suppress both leucocyte rolling and infiltration during EIU. Although EIU is thought to be predominantly an anterior uveitis model, increasing numbers of studies have also demonstrated leucocyte infiltration in the posterior segment of the eye.^{9,24–31} While leucocyte behaviour in the posterior segment might be somewhat different from that in anterior uveitis, it would be helpful to evaluate the sequential steps of leucocyte infiltration to understand the mechanisms of anti-inflammatory agents. In this study, we investigated the anti-inflammatory effects of SKK-60060 on leucocyte rolling in the retinal veins and subsequent leucocyte infiltration into the vitreous cavity. Furthermore, we evaluated the suppressive effects of SKK-60060 on leucocyte infiltration and protein leakage into the aqueous humour.

MATERIALS AND METHODS

Animal model

Animals were handled in accordance with the ARVO statement for the use of animals in ophthalmic and vision research. EIU was induced in female Lewis rats, each weighing

approximately 200 g, by injections of 100 µg of LPS (*Salmonella typhimurium*; Difco Laboratories, Detroit, MI, USA) diluted in 0.1 ml sterile saline into one hind footpad. Control animals received a footpad injection of saline only.

Treatment

In ischaemia reperfusion injury, SKK-60060 has bell-shaped dose dependency with ideal dose of 0.8 mg/kg. In our preliminary experiments using the EIU model, we confirmed that 0.1 mg/kg was sufficient to reduce leucocyte rolling. SKK-60060 (0.1 mg/kg), dissolved in sterile saline, was administered intravenously 15 minutes before LPS injection to experimental animals; vehicle treated rats were given the same volume of saline. Five different rats were used at each time point in both vehicle treated and SKK-60060 treated groups.

Acridine orange digital fluorography

Acridine orange digital fluorography has been described elsewhere.^{32,33} In this technique, a scanning laser ophthalmoscope (SLO; Rodenstock Instruments, Munich, Germany) coupled with a computer assisted image analysis system makes continuous high resolution images of the fundus of animals injected with the metachromatic fluorochrome acridine orange (AO; Wako Pure Chemical, Osaka, Japan). The obtained images are recorded for further analysis on S-VHS videotape at the rate of 30 frames/second.

AO digital fluorography was performed at 6, 12, 24, and 48 hours after LPS injection. Immediately before AO digital fluorography was performed, the rats were anaesthetised with a 1:1 mixture of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg), and the pupils dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. In each rat, a catheter was inserted into the tail vein and arterial blood pressure was monitored with a blood pressure analyser (IITC, Woodland Hills, CA, USA). The rat was then placed on a movable platform, and AO (0.1% solution in saline) was injected through the tail vein catheter. The fundus was observed with the SLO in the 40° field. The behaviour of leucocytes can be observed within several seconds after AO infusion, because the dye circulation time is less than 10 seconds and AO is so membrane permeable that leucocytes become stained shortly after its infusion. Once infused, AO permeates into tissues gradually. Therefore, AO was injected for 1 minute at a rate of 1 ml/min to investigate the behaviour of leucocytes for a few minutes. At 20 minutes after the injection of AO, the fundus was observed again to evaluate leucocyte infiltration into the vitreous cavity.^{10,29}

Image analysis

The video recordings were analysed with an image analysis system consisting of a computer equipped with a video digitiser (Radius, San Jose, CA, USA). The video image was digitised in real time (30 frames/second) to 640 horizontal and 480 vertical pixels with an intensity resolution of 256 steps.

Rolling leucocytes were defined as leucocytes that moved more slowly than free flowing leucocytes. When evaluating the number of rolling leucocytes, a locus 1 disc diameter away from the centre of the optic disc was picked up in each vessel. The number of rolling leucocytes passing through this locus

for 1 minute were counted. This was defined as the number of rolling leucocytes in a vessel. We then calculated the average number of rolling leucocytes in all major veins. This was used as the flux of rolling leucocytes in a given rat. The number of leucocytes infiltrating into the vitreous was determined by counting the number of fluorescent dots in the vitreous within a circle with a radius of 1 disc diameter from the centre of the optic disc.

Diameters of all major vessels were measured at 1 disc diameter from the centre of the optic disc in monochromatic images recorded before AO injection. Each vessel diameter was calculated in pixels as the distance between the half height points determined separately on each side of the density profile of the vessel image. Averages of the arterial and venous diameters were used as the arterial and venous diameters for each individual rat. After the experiments were completed, each rat was killed with an overdose of anaesthesia, and the study eye was enucleated. To determine a calibration factor to convert values measured on a computer monitor (in pixels) into real values (in µm), the retina was flat mounted and then the distance which corresponded to the 640 pixels side of a computer monitor image in the retina was measured under a microscope.

Analysis of leucocytes and protein in aqueous humour

Aqueous humour was collected by anterior chamber puncture using a 27 gauge needle at 6, 12, 24, and 48 hours after LPS injection. For the leucocyte count, the sample was suspended with an equal volume of 0.4% trypan blue stain solution, and the leucocytes were counted under a light microscope. The concentration of protein in aqueous humour was determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) and dilution of bovine serum albumin (Sigma Chemical, St Louis, MO, USA) as a standard.

Leucocyte count in peripheral blood

Blood anticoagulated with EDTA was obtained from the abdominal aorta of each rat after the experiment. The blood sample was analysed using a haematology analyser (Coulter Counter T-890; Coulter Electronics, Tokyo, Japan).

Statistical analysis

All values were expressed as mean (SEM). Student's *t* test was used to compare two groups. To compare three or more conditions, statistical analysis was performed by the analysis of variance with post hoc comparisons tested using Scheffe's procedure. Differences were considered statistically significant when the probability was less than 0.05.

RESULTS

Leucocyte rolling

Table 1 shows the leucocyte count in peripheral blood. SKK-60060 treatment had no significant influence on the peripheral leucocyte count in rats with EIU.

No leucocyte rolling was identified in the major retinal vessels of control rats. In EIU induced rats, although no leucocyte rolling was observed along the major retinal arteries throughout the experiment, some leucocytes were seen to be rolling along the retinal veins. The flux of rolling leucocytes in EIU

Table 1 Peripheral leucocyte count for rats

Treatment	Peripheral leucocyte count ($\times 10^6/l$)				
	Control	6 hours	12 hours	24 hours	48 hours
Vehicle	6.5 (0.4)	2.7 (0.5)	5.2 (1.2)	6.6 (0.4)	5.1 (0.6)
SKK-60060	6.5 (0.4)	2.6 (0.3)	4.7 (0.6)	4.7 (0.9)	6.6 (0.7)

Values are mean (SEM) ($n=6$ at each time point in each group). No significant differences were found between vehicle-treated and SKK-60060 treated rats.

Figure 1 Suppressive effects of SKK-60060 on the flux of rolling leucocytes along the major retinal veins of EIU rats. SKK-60060 or vehicle was administered 15 minutes before the LPS injection. Values are mean (SEM) ($n=6$ at each time point in each group). * $p < 0.01$, compared with vehicle treated rats.

Figure 2 Suppressive effects of SKK-60060 on the number of leucocytes infiltrating into the vitreous cavity in EIU rats. SKK-60060 or vehicle was administered 15 minutes before the LPS injection. Values are mean (SEM) ($n=6$). * $p < 0.01$, † $p < 0.05$, compared with vehicle treated rats.

rats increased gradually after LPS injection and reached its peak at 12 hours. Figure 1 shows the effects of SKK-60060 on

Figure 3 Suppressive effects of SKK-60060 on EIU induced vasodilation in the major retinal arteries (A) and veins (B). SKK-60060 or vehicle was administered 15 minutes before the LPS injection. Values are mean (SEM) ($n=6$ at each time point in each group). * $p < 0.01$, † $p < 0.05$, compared with vehicle treated rats; § $p < 0.01$, § $p < 0.05$, compared with control rats.

the flux of rolling leucocytes along the major retinal veins. SKK-60060 administered 15 minutes before LPS injection significantly inhibited leucocyte rolling, compared with vehicle treated rats ($p < 0.0001$). The flux of rolling leucocytes in SKK-60060 treated rats was reduced by 92.8% ($p = 0.0099$), 80.9% ($p < 0.0001$), 77.8% ($p = 0.0006$), and 87.5% ($p = 0.0001$) at 6, 12, 24, and 48 hours after LPS injection, respectively.

Leucocyte infiltration into vitreous cavity

In control rats, no leucocytes were observed in the vitreous. With the induction of EIU, the number of leucocytes that infiltrated the vitreous cavity was increased from 24 hours after EIU induction, and peaked at 48 hours. However, leucocyte infiltration into the vitreous cavity was significantly suppressed with SKK-60060 treatment ($p < 0.0001$, Fig 2). At 48 hours after LPS injection, the maximum number of leucocytes in the vitreous cavity was reduced by 97.0% in SKK-60060 treated rats compared with vehicle treated rats ($p = 0.0001$).

Major retinal vessel diameters

Figure 3 shows the changes in major retinal vessel diameters in vehicle treated and SKK-60060 treated rats. Both arteries and veins showed substantial vasodilation after EIU induction. In SKK-60060 treated rats, however, vasodilation in arteries and veins was significantly suppressed compared with that in vehicle treated rats ($p = 0.0032$ and $p < 0.0001$, respectively). The diameters of major retinal veins in SKK-60060 treated rats were reduced by 16.4% ($p = 0.0058$), 12.7% ($p = 0.042$), and 23.7% ($p = 0.0011$) at 6, 12, and 24 hours after LPS injection, respectively.

Leucocyte infiltration and protein leakage into aqueous humour

Figure 4 shows the effects of SKK-60060 on leucocyte count and protein concentration in aqueous humour. In vehicle treated rats, both leucocyte count and protein concentration gradually increased after EIU induction and reached a peak level at 24 hours. In SKK-60060 treated rats, however, leucocyte count and protein concentration in aqueous humour were significantly lower than in vehicle treated rats ($p < 0.0001$ for both leucocyte count and protein concentration). At 24 hours after LPS injection, SKK-60060 treatment suppressed leucocyte infiltration in aqueous humour by 92.2% ($p < 0.0001$) and protein leakage by 91.2% ($p < 0.0001$).

DISCUSSION

In the present study, we have demonstrated that SKK-60060 can block leucocyte rolling along the major retinal veins and

Figure 4 Suppressive effects of SKK-60060 on leucocyte infiltration (A) and protein leakage (B) into the aqueous humour induced by EIU. SKK-60060 or vehicle was administered 15 minutes before the LPS injection. Values are mean (SEM) (n=6 at each time point in each group). *p < 0.01, †p < 0.05, compared with vehicle treated rats.

subsequent leucocyte infiltration into the vitreous cavity during EIU in rats. The inhibiting effects of SKK-60060 on the actions of P-selectin and L-selectin would result in suppression of the leucocyte infiltration process. Accumulating evidence indicates that P-selectin mediates the step of leucocyte rolling at sites of inflammation.^{34–36} We have shown the contribution of P-selectin in leucocyte rolling in the retina of EIU rats.¹⁰ The inhibitory effect of SKK-60060 on P-selectin would thus contribute to the suppression of leucocyte rolling in EIU. Since leucocyte rolling is the initial step of leucocyte infiltration, and is a prerequisite for the next steps to infiltration,³⁷ the suppressive effect of SKK-60060 on leucocyte rolling would account at least partially for the attenuation of leucocyte infiltration into the vitreous cavity.

In addition to its inhibitory effect on P-selectin, SKK-60060 suppresses leucocyte rolling and subsequent infiltration by inhibiting the action of L-selectin. L-selectin is constitutively expressed on leucocytes and is thought to mediate initial attachment of leucocytes to endothelial cells followed by P-selectin mediated leucocyte rolling.^{16 38–40} Furthermore, recent studies have shown that L-selectin has a role in the signal transduction for activation of leucocytes to emigrate out of the vasculature,^{14 15} so SKK-60060 might suppress leucocyte infiltration by reducing this activity through inhibition of L-selectin.

In the current study, SKK-60060 significantly suppressed retinal vasodilation in rats with EIU. Although the mechanism of its suppressive effects on vasodilation is not clear, its inhibitory effects on leucocyte-endothelium interactions could account for suppression of the vasodilation. We have shown that P-selectin antibody also suppresses vasodilation of retinal arteries and veins in rats with EIU.¹⁰ The inhibitory effects on leucocyte infiltration would contribute to its suppressive effects on retinal vasodilation, because accumulated leucocytes can produce a large amount of nitric oxide.

Although increasing numbers of studies have demonstrated that LPS causes an inflammatory reaction in the posterior segment of eyes,^{9 24–31} EIU has been thought to be a model of anterior uveitis. Therefore, the inflammatory reaction would be more severe in the anterior uvea. To evaluate the effects of SKK-60060 on anterior uveitis, we studied leucocyte count and protein concentration in aqueous humour. SKK-60060 treatment substantially reduced leucocyte infiltration in the aqueous humour. Because leucocyte rolling in iris vessels is reportedly upregulated during EIU,⁴¹ SKK-60060 should suppress leucocyte rolling in both iris and ciliary vessels, resulting in suppression of leucocyte infiltration into the aqueous humour. Moreover, SKK-60060 successfully suppressed protein leakage into aqueous humour. Attenuation of the process

of leucocyte infiltration might contribute to the protective effects on blood-ocular barrier disruption during EIU, as well as to the suppressive effects of SKK-60060 on vasodilation in the retina. Both number of leucocytes and protein levels in aqueous humour of SKK-60060 treated rats were increasing at 48 hours after LPS injection, while these inflammatory reactions started to subside in vehicle treated rats at 48 hours. The anti-inflammatory effects of SKK-60060 seemed to be weakened at 48 hours compared to the time points of 12 and 24 hours. Effective dose of SKK-60060 might not be able to be maintained after 24 hours.

Compared with the anti-inflammatory effects of inhibiting P-selectin with anti-P-selectin antibody,^{8 10} SKK-60060 showed almost the same degree of inhibition of leucocyte rolling in EIU. Both anti-P-selectin antibody and SKK-60060, at their peaks, suppressed the number of leucocyte rolling in the retinal veins of rats with EIU by about 80%. However, the suppressive effect of SKK-60060 on leucocyte infiltration into the vitreous cavity was greater than that of anti-P-selectin antibody: SKK-60060 suppressed leucocyte infiltration by 97% at its peak, while anti-P-selectin antibody reportedly suppressed leucocyte infiltration into the vitreous cavity by 61%.¹⁰ Moreover, in contrast with the report that anti-P-selectin antibody could not suppress protein leakage into the aqueous humour,⁸ SKK-60060 significantly suppressed protein leakage into aqueous humour. These differences between anti-P-selectin antibody and SKK-60060 in their anti-inflammatory effects are probably because of the ability of SKK-60060 to inhibit L-selectin actions in addition to its inhibitory effects on P-selectin actions. Targeting of a single adhesion molecule might not be sufficient for treatment of uveitis. In the EIU model in mice, Whitcup *et al*⁷ have shown that inhibiting two selectins is necessary to suppress leucocyte infiltration. Similarly, Suzuma *et al*⁹ have demonstrated that blocking two selectins can suppress protein leakage into aqueous humour during EIU in rats.

There are several advantages in using SKK-60060 as an anti-inflammatory agent compared with other agents, such as fucoidin, sulfatide, and monoclonal antibodies, against adhesion molecules. The supply of monoclonal antibodies for human use is limited and repeated administration should be avoided because of their antigenicity if not human derived. Furthermore, decrease in immunological competence against infection may be a major side effect. Fucoidin, a homopolymer of fucose, is known to block both P-selectin and L-selectin function. However, fucoidin is such a unstable agent that continuous administration is needed to suppress the development of inflammation.^{42–44} Sulfate galactosylceramide, which is a good ligand for L-selectin and P-selectin, is difficult to dissolve

and therefore has limited potential as a therapeutic agent. SKK-60060 is chemical synthesised, easily dissolved, is stable in blood, and is not disseminated systemically. Thus it appears to have no major disadvantages but does have major advantages

In conclusion, we have demonstrated that the selectin inhibitor SKK-60060 suppresses leucocyte rolling along major retinal veins, which results in the inhibition of subsequent leucocyte infiltration into the vitreous cavity. It is hypothesised that its inhibitory effects on the actions of P-selectin and L-selectin result in suppression of leucocyte rolling and subsequent infiltration. Taken together with the finding that SKK-60060 suppresses leucocyte infiltration and protein leakage into the aqueous humour, SKK-60060 may have a role in the management of patients with uveitis.

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