**Cornea**

**Blockade of Insulin Receptor Substrate-1 Inhibits Corneal Lymphangiogenesis**

Deniz Hos, Birgit Regenfuss, Felix Bock, Jasmine Onderka, and Claus Cursiefen

**PURPOSE.** To analyze whether insulin receptor substrate (IRS-1) is involved in lymphatic vessel development and whether IRS-1 blockade can inhibit lymphangiogenesis in vivo.

**METHODS.** The impact of IRS-1 blockade by GS-101 (Aganirsen), an antisense oligonucleotide against IRS-1, on lymphatic endothelial cell (LEC) proliferation was assessed by ELISA. Furthermore, the effect of IRS-1 blockade on prolymphangiogenic growth factor expression by LECs and macrophages (peritoneal exudate cells) was tested by real-time PCR. The mouse model of inflammatory corneal neovascularization was used to analyze the effect of IRS-1 blockade in vivo: after corneal suture placement, mice were treated with GS-101 eye drops (twice daily afterwards for 1 week, 5 μL per drop; 50, 100, or 200 μM). Afterward, corneal wholemounts were prepared and stained for blood and lymphatic vessels.

**RESULTS.** Blockade of IRS-1 by GS-101 inhibited LEC proliferation dose dependently. GS-101 led to decreased VEGF-A expression levels in LECs, whereas VEGF-C, VEGF-D, and VEGFR3 showed no significant change. In macrophages, VEGF-A expression levels were also inhibited by IRS-1 blockade. Additionally, GS-101 strongly inhibited macrophage-derived VEGF-C, VEGF-D, and VEGFR3 expression. In vivo, corneal hemangiogenesis was significantly inhibited when used at a concentration of 200 μM (by 17%; P < 0.01). Corneal lymphangiogenesis was significantly inhibited when used at a dose of 100 μM (by 21%; P < 0.01), and the highest used dose (200 μM) showed an even stronger inhibition (by 28%; P < 0.001).

**CONCLUSIONS.** Blockade of IRS-1 inhibits not only hemangiogenesis but also lymphangiogenesis. To the authors’ knowledge, this is the first evidence that IRS-1 is involved in the molecular pathway leading to lymphangiogenesis. (Invest Ophthalmol Vis Sci. 2011;52:5778–5785) DOI:10.1167/iovs.10-6816

Lymphangiogenesis, the development and growth of lymphatic vessels, occurs under a variety of pathologic conditions, such as chronic inflammation and graft rejection, and plays a crucial role in cancer growth and dissemination. This process is driven by the production of prolymphangiogenic growth factors and proinflammatory cytokines such as VEGF-A, VEGF-C, VEGF-D, FGF, TNF-α, and IL-1β. Subsequent binding of these factors to their receptors initiates complex signaling cascades, and the involvement of numerous molecules and many different cell types makes it challenging to fully understand the process of lymphatic vessel development but also offers many therapeutic targets to interfere with and inhibit lymphangiogenesis.

Insulin receptor substrate (IRS-1) is a cytosolic adapter protein without intrinsic kinase activity. The main function of this protein is to recruit other proteins to their receptors and induce the organization of intracellular signaling cascades. IRS-1 was originally isolated as an insulin receptor substrate but has since been shown to work as a proximal scaffold protein in a broad variety of growth hormone and cytokine receptor signaling cascades. The role of IRS-1 in angiogenesis seems to be through its interaction with the VEGF-receptor complex. Furthermore, it has been shown that IRS-1 is able to interact with integrins, multifunctional proteins also involved in lymphangiogenesis.

Several studies have investigated the role of IRS-1 signaling in hemangiogenesis. IRS-1 expression in endothelial cells is upregulated under angiogenic conditions. Furthermore, hypoxic retinal neovascularization is reduced in IRS-1 knockout mice. Moreover, IRS-1 is also expressed in the cornea, and it has recently been shown that GS-101 (Aganirsen), an antisense oligonucleotide that blocks the expression of IRS-1, inhibits corneal hemangiogenesis, both in experimental and clinical settings. Studies addressing the underlying antiangiogenic mechanisms showed that GS-101 inhibited endothelial tube-like structure formation and VEGF-A and IL-1β expression by endothelial cells.

However, it is thus far unclear whether IRS-1 signaling is involved in the molecular pathway leading to lymphatic vessels. Therefore, the aim of this study was to investigate whether the blockade of IRS-1 signaling by GS-101 is also able to suppress lymphangiogenesis. We analyzed the impact of IRS-1 blockade on lymphatic endothelial cell (LEC) proliferation and prolymphangiogenic factor expression by LECs and macrophages, which have been shown to be essential mediators of lymphangiogenesis. Finally, we analyzed the effect of GS-101 eye drops on inflammatory corneal lymphangiogenesis in vivo.

**METHODS**

**Lymphatic Endothelial Cell Culture, Treatment, and Proliferation ELISA**

LEC proliferation ELISA was used, with slight modifications, as previously described. Briefly, human lymphatic microvascular endothelial cells (Cambrex Bio Science, Walkersville, MD) were cultured in EGM2-MV full medium (EGM 2-MV full medium contains endothelial cell growth factors such as VEGF and bFGF). For ELISA, cells were seeded in a 96-well plate in EGM2-MV medium at a density of 4 × 10⁴ cells/well. Six hours after seeding, medium was replaced with EGM2-MV minimal medium (without growth factors), BrdU (10 μL/mL;...
Cell Proliferation ELISA, BrdU, Roche, Indianapolis, IN), and GS-101 at various concentrations were then added. Cells were fixed and stained after 48 hours according to manufacturer’s instructions. Colorimetric analysis was performed with an ELISA reader (Multiskan Spectrum; Thermo Electron Corporation, Waltham, MA). The mean extinction of the control wells was defined as 100%; the extinction of all wells was then related to this value (LEC proliferation ratio). For RNA expression analyses, LECs were incubated in RPMI 1640 medium containing various concentrations of GS-101 for 24 hours, followed by RNA extraction.

Collection, Culture, and Treatment of Peritoneal Macrophages

Thioglycollate-induced peritoneal exudate cells (PECs) were collected from the peritoneal cavities of 8- to 10-week-old female mice, as described previously.22 PECs were washed, resuspended, and cultured at 37°C in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. After adhesion, nonadherent cells were removed by washing with culture medium, and adherent cells were then used as macrophages. Cells collected by this method are F4/80 

\(^{22}\) (both 90%), and CD11b (both 99%).

RNA Expression Analyses

For RNA expression analyses, cells were incubated in RPMI 1640 medium containing various concentrations of TNF-α, IL-1β (both 10 ng/mL), or GS-101 for 24 hours, followed by RNA extraction.

RNA Isolation and Real-Time PCR

RNA from LECs and PECs was isolated with an RNA purification kit (RNeasy Micro Kit; Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized with random hexamers using reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA). Primer (MWG Biotech, Ebersberg, Germany) was designed using Primer3 software and BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information). PCR reactions (20 µL) contained 10 to 20 ng cDNA (depending on the analyzed gene), 0.4 M of each forward and reverse primer, and master mix (SoFast EvaGreen Supermix; Bio-Rad, Hercules, CA). Real-time PCR was performed under the following conditions: initial denaturation step of 95°C for 2 minutes, 40 cycles of 95°C for 5 seconds and of 56°C to 62.4°C (depending on the analyzed gene) for 15 seconds, followed by an additional denaturation step of 95°C for 1 minute. The data were analyzed using the comparative Ct method (2\(^{-\Delta\Delta C\text{t}}\)).

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<th>Table 1. Primers Used for Real-Time PCR</th>
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F, forward; R, reverse.
step of 95°C for 60 seconds and a subsequent melt curve analysis to check amplification specificity. All PCR products were analyzed by gel electrophoresis on a 2% agarose gel and were visualized by ethidium bromide staining. Primer sequences, product sizes, and respective annealing temperatures are summarized in Table 1.

Real-time PCR results were analyzed by the comparative threshold cycle method with human HPRT1 (or mouse HPRT, respectively) as the endogenous reference gene for all reactions. The relative messenger RNA (mRNA) level in the untreated group was used as the normalized control for the treatment groups. All assays were conducted three times and performed in triplicate; a nontemplate control was included in all the experiments to evaluate DNA contamination of the reagents used.

Animals and Anesthesia

All animal protocols were approved by the local animal care committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with intraperitoneal injection of a combination of ketamine (Ketanest S; 8 mg/kg) and xylazine (Rompun; 0.1 mL/kg). All mice were 8- to 10-week-old females (purchased from Charles River Laboratories, Wilmington, MA).

Suture-Induced, Inflammatory Corneal Neovascularization Assay

The mouse model of suture-induced inflammatory corneal suture placement was used as previously described.19 Before corneal neovascularization, each animal was deeply anesthetized. The healthy cornea actively maintains its avascularity, and, especially, the corneal epithelium contains antiangiogenic molecules such as soluble VEGF receptor 3.23,24 Therefore, to obtain higher angiogenic responses, the central cornea was marked with a 2-mm diameter trephine and deepithelialized before suture placement. Three 11–0 nylon sutures (Serag Wiessner, Naila, Germany) were then placed intrastromally, with two stromal incursions each extending over 120° of corneal circumference. The outer point of suture placement was chosen near the limbus, and the inner suture point was chosen toward the central cornea equidistant from the limbus to obtain standardized angiogenic responses. Sutures were left in place for 7 days. Treatment groups received GS-101 eye drops (twice daily for 1 week, 5 μL per drop; 50, 100, or 200 μM). Control mice received equal amounts of saline solution. After 1 week, mice were killed and corneas were prepared. The corneal neovascularization assay included 18 mice per group.

Corneal Wholemounts and Morphologic Determination of Hemangiogenesis and Lymphangiogenesis

The excised corneas from the corneal neovascularization assay were rinsed in PBS and fixed in acetone for 20 minutes. After washing and blocking with 2% bovine serum albumin (BSA) in PBS for 2 hours, corneas were stained overnight at 4°C with a rabbit anti-mouse LYVE-1 antibody (Abcam, Cambridge, MA) and FITC-conjugated rat anti-mouse CD11b antibody (SeroSite, Raleigh, NC). On day 2, IRS-1 was detected with a Cy3-conjugated secondary goat anti-rabbit antibody (Dianova). Corneas were moved to slides (Superfrost), covered with fluorescent mounting medium (DAKO), and stored at 4°C in the dark.

Statistical Analysis

Statistical analyses were performed (Excel 2000; Microsoft, Redmond, CA) and InStat 3 Version 3.06 [GraphPad Software Inc., San Diego, CA]. Statistical significance was determined using the Student’s t-test. For the comparison of more than two groups or the analysis of dose-dependent responses, statistical significance was determined using one-way analysis of variance test (ANOVA). P < 0.05 was considered statistically significant. Graphs were drawn using biostatistics/curve fitting/scientific graphing software (Prism4, version 4.03; GraphPad Software Inc).

RESULTS

IRS-1 Signaling Blockade by GS-101 Inhibits Lymphatic Endothelial Cell Proliferation

We analyzed the impact of IRS-1 signaling blockade on LEC proliferation in vitro. LECs were exposed to increasing concentrations of GS-101; proliferation was then assessed by ELISA. Incubation with GS-101 significantly suppressed LEC proliferation dose dependently. GS-101 (10 μM) inhibited LEC proliferation by 19% (P < 0.001), whereas 20 μM GS-101 led to an inhibition of LEC proliferation by 46% (P < 0.001). Higher concentrations had no additional inhibitory effect (40 μM GS-101, inhibition by 48%; 20 μM vs. 40 μM; P > 0.05; **P < 0.001; data are expressed as mean ± SEM).

Figure 1. IRS-1 blockade by GS-101 inhibits LEC proliferation in vitro. LECs were exposed to increasing concentrations of GS-101, an anti-sense oligonucleotide against IRS-1; proliferation was then measured by ELISA. Treatment of LECs with GS-101 significantly suppressed cell proliferation. 10 μM GS-101 inhibited LEC proliferation by 19%, whereas 20 μM GS-101 led to an inhibition of LEC proliferation by 46%. Higher concentrations had no additional inhibitory effect (40 μM GS-101, inhibition by 48%; 20 μM vs. 40 μM; P > 0.05; **P < 0.001; data are expressed as mean ± SEM).
concentrations had no additional inhibitory effect (40 μM GS-101, inhibition by 48% \( P < 0.001 \); 20 μM vs. 40 μM, not significant \( P > 0.05 \); Fig. 1).

Blockade of IRS-1 in LECs Impairs Expression of VEGF-A but Not of VEGF-C, VEGF-D, or VEGFR3

LECs were incubated with various concentrations of GS-101 for 24 hours. mRNA expression levels of VEGF-A, VEGF-C, VEGF-D, and VEGFR3 were then measured by real-time PCR. VEGF-A expression was inhibited dose dependently: 10 μM GS-101 led to an inhibition of VEGF-A mRNA levels by 14% \( P < 0.05 \), and 20 μM inhibited VEGF-A expression by 34% \( P < 0.001 \); Fig. 2A). However, VEGF-C and VEGF-D expression levels presented no significant change. VEGF-C expression levels showed a slight, but not significant, decrease (10 μM, reduction by 14% \( P = 0.084 \); 20 μM, reduction by 13% \( P = 0.063 \); Fig. 2B), and VEGF-D levels, which were very low, remained unaffected (10 μM, \( P = 0.41 \); 20 μM, \( P = 0.31 \); Fig. 2C). VEGFR3 levels also remained unaltered (10 μM, \( P = 0.85 \); 20 μM, \( P = 0.34 \); Fig. 2D).

IRS-1 Is Significantly Involved in Pro(lymph)angiogenic Growth Factor Expression by Macrophages

To analyze whether macrophages express IRS-1 and whether IRS-1 is regulated under inflammatory conditions, PECs were incubated in medium alone or with the addition of the proinflammatory cytokines TNF-α or IL-1β. PCR analyses revealed that IRS-1 is expressed in PECs, but the addition of either TNF-α or IL-1β led to a considerable change in IRS-1 expression levels. However, the addition of GS-101 significantly suppressed IRS-1 expression in PECs (Fig. 3A).

To analyze the potential involvement of IRS-1 signaling in macrophage-derived growth factor expression, PECs were incubated with GS-101. mRNA expression levels of VEGF-A, VEGF-C, VEGF-D, and VEGFR3 were then measured by real-time PCR. Because our experiments had shown that IRS-1 seems not to be upregulated in PECs under inflammatory conditions, we decided to analyze the effect of IRS-1 blockade only on basal growth factor expression by PECs without previous

![Figure 2](https://iovs.arvojournals.org/)
stimulation. Treatment with GS-101 inhibited VEGF-A expression dose dependently: 10 μM GS-101 inhibited VEGF-A mRNA levels by 11% (P < 0.05), and 20 μM GS-101 inhibited VEGF-A expression by 23% (P < 0.01; Fig. 3B). Notably, VEGF-C expression levels were strongly inhibited by IRS-1 blockade: 10 μM GS-101 led to a decrease in VEGF-C expression levels by 70% (P < 0.001), and 20 μM of GS101 inhibited VEGF-C expression by 64% (P < 0.001; Fig. 3C). VEGF-D expression was also inhibited, albeit only when GS-101 was used at a dose of 10 μM (reduction by 27%; P < 0.01). The inhibition of VEGF-D expression was no longer detectable when GS-101 was used at the higher dose of 20 μM (P > 0.05; Fig. 3D). VEGFR3 expression levels also showed a decrease: 10 μM GS-101 led to a reduction of VEGFR3 expression by 33% (P < 0.05), and the addition of 20 μM GS-101 led to an inhibition by 50%, P < 0.001; Fig. 3E).

Blockade of IRS-1 Signaling Inhibits Hemangiogenesis and Lymphangiogenesis In Vivo

The healthy cornea lacks both blood vessels and lymphatic vessels, but it can secondarily be invaded by both vessel types after severe inflammation. For that reason, the cornea is widely used to study mechanisms of hemangiogenesis and lymphangiogenesis.26–28 To analyze the value of IRS-1 signaling in vivo, we used the suture-induced corneal neovascularization assay as a well-established and accepted model for induction and analysis of inflammatory corneal hemangiogenesis and lymphangiogenesis.19,29,30 Blockade of IRS-1 after suture placement led to a significant reduction of inflammatory corneal neovascularization in vivo. When GS-101 was used at the highest dose of 200 μM, corneal hemangiogenesis was significantly inhibited (by
17% in comparison with control animals; \( P < 0.01 \). Lower concentrations of GS-101 eye drops had no significant effect on blood vessel growth \( (P > 0.05) \). Corneal lymphangiogenesis was already significantly inhibited by 21% when used at a dose of 100 \( \mu M \) \( (P < 0.01) \), and the highest dose (200 \( \mu M \)) showed an even stronger inhibition (26\% less; \( P < 0.001 \)). When used at the lowest dose of 50 \( \mu M \), GS-101 eye drops did not show a significant inhibition of corneal lymphangiogenesis \( (P > 0.05) \); Fig. 4).

**Corneal Macrophages Express IRS-1**

We stained corneas for CD11b and IRS-1 to analyze whether corneal macrophages also express IRS-1. Figure 5 shows that some, but not all, corneal CD11b\(^+\) cells express IRS-1. The expression of IRS-1 by corneal macrophages in vivo, together with the impact of IRS-1 blockade on macrophage-derived growth factor expression in vitro, led us to the conclusion that the inhibition of macrophage-derived IRS-1 expression by GS-101 might indeed be one of the mechanisms contributing to fewer corneal lymphatic vessels in vivo.

**DISCUSSION**

Research on lymphangiogenesis is an emerging field, and our knowledge of the mechanisms underlying the formation of new lymphatic vessels is steadily expanding. However, the importance of all molecules involved in this process is still not fully understood. Several studies have investigated the role of...
IRS-1 in *bem*angioiogenesis, 15–18 but the value of IRS-1 in lymphangiogenesis was thus far not clear. We showed in this study that IRS-1 signaling is also involved in the development of new lymphatic vessels and that the blockade of IRS-1 expression by GS-101 inhibits not only corneal hemangiogenesis but also lymphangiogenesis in vivo.

Inhibition of hemangiogenesis by GS-101 started at a dose of 200 μM, whereas inhibition of lymphangiogenesis started at even lower concentrations, beginning at 100 μM. The significant inhibition of corneal lymphatic vessel growth by GS-101 let us conclude that IRS-1 also has an important role in lymphangiogenesis, with an even stronger impact of its downregulation on lymphatic vessel growth than on blood vessel growth.

It has previously been shown that GS-101 is able to inhibit endothelial tube-like structure formation in human umbilical vein endothelial cells. 17 However, the impact of GS-101 on direct proliferation of lymphatic endothelial cells was thus far not addressed. Blockade of IRS-1 expression by GS-101 inhibited LEC proliferation dose dependently, with maximal inhibition at 20 μM. There was no additional benefit of higher concentrations, which probably indicates a saturation of IRS-1 inhibition. This is in line with previous results showing that 20 μM GS-101 is sufficient to minimize IRS-1 expression in endothelial cells. 17 Additionally, it was previously shown that IRS-1−/− mice develop only 40% less hypoxia-induced retinal neovascularization, 19 demonstrating that IRS-1 is notably involved, but not essential, for angiogenesis.

Furthermore, GS-101 leads to reduced expression levels of VEGF-A and IL-1β in endothelial cells. 17 Besides VEGF-A, we analyzed the effect of GS-101 on the expression levels of VEGF-C, VEGF-D, and VEGFR3 in lymphatic endothelial cells. We could also detect a dose-dependent inhibition of VEGF-A expression. However, neither VEGF-C nor VEGF-D expression was significantly affected by IRS-1 blockade. VEGF-C expression levels showed a slight, but not yet significant, decrease, and VEGF-D levels remained unaffected. This could have been due to different regulatory pathways among the various VEGF members. However, VEGF-A is the VEGF member with the highest expression levels even in lymphatic endothelial cells, and it is known that VEGF-D in particular is just barely detectable in endothelial cells. 31 Therefore, we cannot rule out the possibility that we could not identify inhibition caused by already low growth factor levels.

In addition to endothelial cells, macrophages also strongly contribute to lymphangiogenesis. 19, 20 It was shown that CD11b+ cells are able to form vessel-like tubes and to integrate into preexisting lymphatic vessels. 20 Furthermore, a multitude of proangiogenic growth factors are secreted by macrophages, leading to a strong augmentation of both hemangiogenesis and lymphangiogenesis. 19 It was previously shown that downregulation of IRS-1 signaling seems to be associated with a decrease in the number of infiltrating macrophages. 16 However, the effect of IRS-1 blockade on pro(lymph)angiogenic factor production by macrophages was not investigated. We could detect a significant downregulation of VEGF-A expression after treatment of macrophages with GS-101. Additionally, VEGF-C expression was strongly suppressed by GS-101, and expression levels of VEGF-D decreased, albeit only after treatment with 10 μM GS-101. Surprisingly, when GS-101 was used at the higher dose of 20 μM, inhibition was no longer detectable. Several studies have reported differential and even paradoxical regulations of the various VEGF members. Moffat et al. 32 demonstrated that tumor cells underexpressing VEGF-A showed higher levels of VEGF-D. On the other hand, O-Charoenrat et al. 33 showed that several growth factors that upregulate VEGF-A lead to a downregulation of VEGF-D expression levels. This could also be in line with our results: high doses of GS-101 might reduce VEGF-A expression below a certain threshold, which then possibly antagonizes the (direct) impact of GS-101 on VEGF-D and, therefore, leads to a subsequent loss of inhibition. Certainly, further investigation is needed to provide evidence for this rather speculative hypothesis.

Altogether, we conclude that IRS-1 blockade seems to suppress a variety of processes leading to the development of new lymphatic vessels. One of the early steps in GS-101 action appears to be inhibition of the number of infiltrating macrophages, as described previously. 16 It is known that macrophages promote lymphangiogenesis in two different ways, either by stimulating preexistent lymphatic endothelial cells or by transdifferentiating and directly forming new lymphatic vessels. This decisive role of infiltrating macrophages, especially in the development of lymphatic vessels, could be a possible explanation of the earlier inhibition of lymphangiogenesis (starting at a dose of 100 μM) rather than of hemangiogenesis (starting at a dose of 200 μM) observed in our in vivo experiments. It is also known that GS-101 diminishes the overall expression of several angiogenic growth factors in the cornea. 16 Moreover, as shown in our study, the quantity of growth factors expressed per macrophage also decreases after IRS-1 blockade. This might be another explanation for a stronger inhibition of lymphangiogenesis given that macrophages are known to secrete several factors specific for lymphangiogenesis but not for hemangiogenesis, namely VEGF-C and VEGF-D, whereas most of the factors leading to blood vessel growth also promote lymphatic vessel growth, such as VEGF-A. Additionally, IRS-1 directly impairs endothelial cell function. It is the task of further investigation to analyze whether lymphatic endothelial cells are more susceptible than blood endothelial cells to IRS-1 blockade.

In summary, we have shown that the blockade of IRS-1 expression by GS-101 inhibits not only corneal hemangiogenesis but also lymphangiogenesis. The effects of GS-101 action seem to occur through its direct interaction with lymphatic endothelial cells, namely proliferation inhibition and VEGF-A expression. Furthermore, IRS-1 blockade impairs lymphangiogenesis indirectly by reducing macrophage-derived growth factor expression (VEGF-A and, especially, VEGF-C). This is, to our knowledge, the first evidence that IRS-1 signaling is involved in the molecular pathway leading to lymphangiogenesis.

**Acknowledgments**

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**References**


