The interleukin-1β modulator gevokizumab reduces neointimal proliferation and improves reendothelialization in a rat carotid denudation model

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A B S T R A C T

Objective: Excessive neointima formation often occurs after arterial injury. Interleukin-1β (IL-1β) is a potent pleiotropic cytokine that has been shown to regulate neointimal proliferation. We investigated the effects of the IL-1β modulator gevokizumab in a rat carotid denudation model.

Methods: Sprague–Dawley rats were subjected to balloon denudation of the right carotid artery and were then randomized to receive a single subcutaneous infusion immediately after balloon injury of saline (control group, n = 13) or gevokizumab (gevokizumab groups, n = 15 in each group: 1, 10 and 50 mg/kg). We evaluated the treatment effects on carotid intima-media thickness (IMT) using ultrasonography, on endothelial regrowth using Evans Blue staining and on inflammatory response using histology. We also assessed the effects of IL-1β and gevokizumab on human umbilical vein endothelial cells (HUVEC) and rat smooth muscle cells.

Results: We found that carotid IMT, in the proximal part of the denuded artery at day 28, was decreased by gevokizumab 1 mg/kg compared with controls. Neointima area and the intima/media area ratio were both reduced in the gevokizumab 1 mg/kg-treated group. Gevokizumab at the 1 mg/kg dose also improved endothelial regrowth. No effect was observed with gevokizumab 10 or 50 mg/kg. Gevokizumab also decreased the inflammatory effect of IL-1β in in vitro cell experiments and protected HUVECs from IL-1β’s deleterious effects on cell migration, apoptosis and proliferation.

Conclusion: A single administration of gevokizumab 1 mg/kg improves endothelial regrowth and reduces neointima formation in rats following carotid denudation, at least in part through its beneficial effects on endothelial cells.

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1. Introduction

Arterial endothelial denudation following angioplasty leads to restenosis, corresponding to an accumulation of inflammatory cells resulting in the proliferation of smooth muscle cells (SMC) which form neointimal tissue at the injury site [1]. Inhibition of proinflammatory pathways has been shown to decrease neointimal formation, highlighting the role of post-denudation inflammation in vascular responses [2,3].

Interleukin-1β (IL-1β) is a potent proinflammatory cytokine secreted by different cell types including macrophages and endothelial cells [4], playing an important role in the pathophysiology of neointima formation [5,6]. In the absence of the natural antagonist of the IL-1 receptor, post-injury intimal proliferation is increased as demonstrated in IL-1Ra-deficient mice [7]. IL-1β mediates the up-regulation of adhesion molecule expression on endothelial cells and smooth muscle cells [8,9], thereby increasing recruitment and extravasation of inflammatory cells, particularly monocytes, and activation of macrophages via the activation of the inflammasome...
Importantly, the inflammasome can in turn induce IL-1β production [11]. Beyond neointima formation, atherosclerotic plaque formation is decreased in animal models by genetic disruption of the IL-1β gene [12] or IL-1 receptor (IL-1R) [13] or pharmacological inhibition [14]. Therefore, our main hypothesis is that modulating IL-1β bioactivity using a biological drug specifically targeting IL-1β (an anti-IL-1β antibody) could favorably affect reendothelialization and neointimal proliferation after arterial denudation, possibly by reducing systemic and local inflammation (hs-CRP, IL-6, TNF-α) and also by regulating expression of adhesion molecules such as VCAM-1 and ICAM-1.

Many therapies targeting IL-1 signaling have been developed to target auto-inflammatory diseases and more recently cardiovascular diseases [15]. Gevokizumab, a potent anti-IL-1β neutralizing antibody, binds to a unique IL-1β epitope that is proximal to, but does not overlap with the receptor/ligand interface. Gevokizumab binding to IL-1β may reduce the rate of assembly of the active signaling complex rather than prohibit its assembly entirely [16]. Gevokizumab is therefore considered a modulator and not a blocker of IL-1β signaling, which may result in the reduction of pathologically high activity while allowing homeostatic signaling in biologically important pathways [17]. Gevokizumab was recently shown to reduce atherosclerotic plaque formation in ApoE-deficient mice [18].

In the current study, we investigated the effects of three different dosages (1, 10 and 50 mg/kg) of a single infusion of this biological drug gevokizumab on reendothelialization and neointimal formation in a rat carotid denudation model.

2. Materials and methods

2.1. Animals and experiments

Animal care and procedures complied with the Canadian Council on Animal Care guidelines and were approved by the institutional ethics committee for animal research. Fifty-eight (58) male Sprague–Dawley rats (3 months of age, weighing 330–360 g) were fed a 0.5% cholesterol-enriched diet starting 2 days before the injury, throughout the experimental protocol. Animals were anesthetized with continuous isoflurane (Abbott, Montreal, Canada) administration (induction 2.5%–3.0%, 1.5 L/min oxygen, followed by 1.5–2.0%, 2 L/min oxygen) and heparin (100 IU in each animal) was injected into the right jugular vein. To maintain analgesia, buprenorphine (100 μg/kg, Schering-Plough, Hertfordshire, UK) was administered i.v. before the surgery and given once daily for 3 days after surgery. Electrocardiogram was monitored and thermoregulation was achieved during the procedure.

The right common, external and internal carotid arteries were exposed by a neck incision under a stereo-microscope (Leica M80, Leica Microsystems, Wetzlar, Germany). Meanwhile, blood flow was temporarily interrupted by ligation of the common and internal carotid arteries using surgical thread. The external carotid artery was partially cut at about 2 mm distally from the arterial bifurcation with micro-scissors. A balloon angioplasty catheter (balloon diameter 1.5 mm, length 15 mm, Maverick, Medtronic, Minneapolis, MN, USA) was introduced through the cut of the external carotid into the common carotid artery and was advanced to the proximal edge of the omohyoid muscle. The balloon was then inflated with saline and rotated three times to ensure uniformity of the extent of balloon injury. The balloon was then deflated and removed. The external carotid artery was ligated and blood flow was restored through the common and internal carotid arteries. Rats were then randomly assigned to receive a single subcutaneous injection of either isotype IgG2 control preparation at the dose of 10 mg/kg (control group, n = 13) or gevokizumab at the dose of 1 mg/kg (gevokizumab 1 mg/kg group, n = 15) or 10 mg/kg (gevokizumab 10 mg/kg group, n = 15) or 50 mg/kg (gevokizumab 50 mg/kg group, n = 15) immediately after balloon injury. Ultrasonographic examinations of both left and right carotid arteries were performed at baseline, at day 14 and day 28 post-denudation. Animals were weighed regularly throughout the experimental protocol. After the final ultrasonogram, rats were sacrificed by exsanguination under anesthesia 28 days after vascular injury. Right carotid arteries were harvested for histological analyses. Blood samples were obtained through the abdominal aorta during exsanguination.

2.2. Gevokizumab

Gevokizumab aliquots were provided by Institut de Recherches Internationales Servier (Suresnes, France) and were kept at 4 °C (see Supplementary methods for details).

2.3. Ultrasonographic determination of carotid intima-media thickness

B-mode ultrasound imaging of carotid arteries was performed at baseline before carotid injury, 14 and 28 days after the injury. Special care was taken to acquire similar imaging during follow-up studies by the same experienced operator. Six cardiac cycles were used to obtain mean values for all measurements, with the operator being blinded to treatment assignment (see Supplementary methods for details).

2.4. Evans blue staining and determination of endothelial regrowth

Endothelial regrowth was evaluated by Evans blue staining. Twenty-eight days after carotid denudation and gevokizumab or control treatment, 5 rats/group received an intravenous injection of 5% Evans blue dye solution in the caudal vein (50 mg/ml/kg; Sigma, Saint Louis, MO, USA) 5 min before sacrifice (see Supplementary methods for details).

2.5. Histomorphometry

The intima and media cross-sectional areas of the carotid arteries were measured and the intima/media area ratios were calculated. All measurements were performed by two experienced investigators blinded to randomized treatment assignment (see Supplementary methods for details).

2.6. Immunohistochemistry

We assessed the levels of leucocytes (CD45), monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) on carotid paraffin-embedded sections (see Supplementary methods for details).

2.7. Blood analyses

Serum soluble intercellular adhesion molecule-1 (sICAM-1) levels were measured at baseline and day 28 after carotid injury by enzyme-linked immunosorbent assay (ELISA) (see Supplementary methods for details). Total cholesterol and triglyceride levels were measured with an automated filter photometer system (Dimension RxL Max; Dade Behring, Deerfield, IL, USA).
2.8. mRNA quantification

An additional group of normal animals (no surgery nor gevokizumab treatment, \( n = 6 \)) was added for RNA analyses (see Supplementary methods for details).

2.9. Cell culture experiments

Primary rat vascular smooth muscle cells (SMC) were isolated from aortas of male Sprague–Dawley rats. Human umbilical vein endothelial cells (HUVECs) and SMCs were treated with IL-1\( \beta \) in presence or absence of gevokizumab or the IgG2 control antibody. Endothelial cell migration was evaluated using an in vitro scratch wound assay. Proliferation was assessed with an MTT assay. Flow cytometry was used to quantitate the apoptosis rate and the expression of adhesion molecules using anti-human E-selectin and VCAM-1 antibodies (BD Biosciences). mRNA expression was assessed by qPCR (see Supplementary methods for details).

2.10. Statistical analyses

All analyses were performed with SAS version 9.3 or higher (SAS Institute Inc., Cary, NC, USA) and conducted at the 0.05 significance level. Continuous variables are presented as adjusted
We evaluated the re-endothelialization of the denuded portion of the carotid artery by Evans blue staining (Fig. 2a). Endothelial regrowth was significantly improved by 3-fold in the gevokizumab 1 mg/kg-treated group compared with controls (31.3 ± 2.8% vs 10.8 ± 2.8%, *P = 0.009) (Fig. 2b). Given the improved endothelial regrowth in the gevokizumab 1 mg/kg-treated group, we assessed neointimal tissue formation using morphometric analysis.

3.4. Low-dose gevokizumab reduces neointimal tissue formation

We assessed neointimal tissue formation on HPS-stained sections at 5 different levels in the proximal half of the injured carotid artery (Fig. 3a). Neointimal area was reduced by 25% in the gevokizumab 1 mg/kg-treated group compared with controls (162,375 ± 14,195 vs 217,601 ± 14,303 μm², *P = 0.016) (Fig. 3b). In support of these findings, gevokizumab 1 mg/kg treatment led to a significant decrease of the intima/media area ratio assessed by morphometry as compared with controls (1.19 ± 0.11 vs 1.63 ± 0.14, *P = 0.015) (Fig. 3c), corresponding to a reduction of 27%.

3.5. Impact of gevokizumab on inflammation

Inflammation is one of the main determinants of intimal proliferation [19]. We therefore evaluated systemic as well as in situ inflammation. In situ, the total number of cells was quantified in the neointimal tissue of HPS-stained sections at 5 different levels in the proximal half of the injured carotid artery. Compared with controls, the total number of cells per section was significantly reduced in the gevokizumab 1 mg/kg-treated group (P = 0.006) as well as in the 50 mg/kg-treated group (P = 0.049) (Fig. 4a).

There was a 56% reduction in neointimal CD45-positive cell surface in the gevokizumab 1 mg/kg-treated group compared with controls (Fig. 4b), which did not reach statistical significance (P = 0.23), probably due to the small sample size (n = 10 and 8, respectively). Gevokizumab treatment did not have an effect on the levels of proteins involved in the IL-1 inflammatory pathways, including VCAM-1 and MCP-1 (data not shown). We also evaluated sICAM-1 and did not find any significant difference between treated groups (data not shown).

Finally, we evaluated changes in the expression of inflammatory and adhesion genes using qRT-PCR. We assessed the expression of the following genes in a carotid segment from normal animals and in the distal half of the injured carotid artery of control and gevokizumab-treated animals: Ccl2, Icam1, Vcam1, Tnf, Il6, Il-1β, Il-1Ra, and Il11r1. Although the expression of most of the inflammatory genes tested (except ICAM-1 and VCAM-1) was significantly increased compared with normal animals, there were no differences observed following treatment (Supplementary Fig. 2). We did not find any differences in the circulating levels of inflammatory cytokines (IL-1β, TNF-α, IL-6, data not shown).

3.6. In vitro assessment of the anti-inflammatory effects of gevokizumab

To further assess the efficacy of gevokizumab and to appraise whether the effects observed in vivo may have been due to its anti-inflammatory effects on endothelial or smooth muscle cells, we treated HUVECs and rat SMCs with IL-1β in presence or absence of gevokizumab and measured the expression of adhesion molecules responsible for inflammatory cell infiltration.

In the experiments using HUVECs, cell exposure to gevokizumab alone or an IgG2 antibody alone did not exert any effect on the cell surface expression of E-selectin or VCAM-1 as assessed by flow
cytometry (data not shown). As expected, exposure of HUVECs to IL-1β induced an increase in expression of E-selectin and VCAM-1 ($P < 0.01$ and $P < 0.05$, respectively, Fig. 5). The IL-1β-induced increase in HUVEC expression of E-selectin was inhibited by both low and higher doses of gevokizumab ($P < 0.01$, Fig. 5a), whereas for VCAM-1, this overexpression was numerically decreased by a low dose of gevokizumab (0.5 ng/mL, $P = 0.063$) and significantly decreased with higher doses (5 ng/mL and 50 ng/mL, $P < 0.01$).

In rat SMC experiments, gevokizumab alone or the IgG2 control antibody alone did not exert any significant effect on the mRNA expression for ICAM-1 and VCAM-1 (data not shown). IL-1β induced a significant overexpression of the mRNAs for ICAM-1 and VCAM-1 (Supplementary Fig. 3), which was significantly decreased by gevokizumab (5 ng/mL or 50 ng/mL, $P < 0.001$, Supplementary Fig. 3).

3.7. In vitro assessment of deleterious effects of IL-1β on endothelial cell migration, apoptosis and proliferation and their blockade by gevokizumab

To assess the effect of IL-1β on endothelial cell migration, we performed a scratch wound assay where HUVECs were exposed to IL-1β. As presented in Fig. 6, wound closure was inhibited by 25.1% by IL-1β alone ($p < 0.0001$) and by 16.4% for IL-1β in presence of control IgG2 antibody ($p = 0.0013$); in contrast, there was no significant difference between control cells (not exposed to IL-1β or gevokizumab) and cells exposed to both IL-1β and gevokizumab. Gevokizumab improved IL-1β-inhibited endothelial cell migration ($P = 0.0021$) whereas the IgG2 control antibody did not exert a significant effect. Direct comparison of the percent difference to control cells between cells exposed to both IL-1β and gevokizumab and those exposed to IL-1β and IgG2 control antibody almost reached statistical significance ($p = 0.0573$, not represented in Fig. 6a).

Exposure of HUVEC cells to IL-1β resulted in other deleterious effects by increasing the percentage of cells undergoing apoptosis. Fig. 6b shows that IL-1β caused a 2.2-fold increase in late apoptosis of HUVECs whereas gevokizumab blocked 53% of this increase ($p = 0.0027$). When HUVECs were treated with the IgG2 control antibody, there was no effect on IL-1β-induced apoptosis. The effects of treatments with gevokizumab and IgG2 control antibody on IL-1β-induced apoptosis were significantly different ($P = 0.0006$).
Conversely, IL-1β decreased significantly the proliferation of HUVECs when compared to unexposed control cells ($P = 0.0167$, Fig. 6c). The addition of the IgG2 control antibody did not exert any significant effect on IL-1β-induced inhibition of proliferation. In contrast, gevokizumab improved significantly the proliferation of HUVECs exposed to IL-1β ($P = 0.0021$). There was also a significant difference between the cells exposed to IL-1β in presence of gevokizumab versus those in presence of the control IgG2 antibody ($P = 0.0038$).

4. Discussion

We have shown that a single infusion of the biological drug specifically targeting IL-1β gevokizumab at 1 mg/kg consistently improves endothelial regrowth, decreases neointimal formation and reduces intima-media thickness in a rat carotid denudation model. In vitro experiments show that gevokizumab may decrease the IL-1β-induced expression of several inflammatory mediators and adhesion molecules by both endothelial and smooth muscle cells. Additionally, the in vitro experiments also demonstrated deleterious effects of IL-1β on endothelial cell migration, apoptosis and proliferation which could be significantly attenuated by gevokizumab. Our findings underline the critical role of IL-1β signaling in re-endothelialization and neointima proliferation and highlight a new strategy to treat injured arteries.

The anti-IL-1β treatment in this rat model of carotid denudation had a major impact on re-endothelialization, which was found to be 2.9 times more complete in the gevokizumab 1 mg/kg-treated group compared with controls. As supported by our in vitro experiments, the local production of IL-1β after carotid denudation has deleterious effects on endothelial cell migration and proliferation/apoptosis and these effects can be alleviated by gevokizumab. The benefit of gevokizumab on re-endothelialization compares favorably with that of other interventions with a positive impact on endothelialization in rat carotid injury models such as local VEGF delivery [20] or treatment with the phosphodiesterase inhibitor cilostazol [21]. In vitro experiments demonstrated that gevokizumab can exert powerful anti-inflammatory effects on both endothelial and smooth muscle cells. Reduced expression of VCAM-1 and inflammatory mediators could result in a more physiologic re-endothelialization associated with a healthier endothelium: for instance, in an in vitro study on migration of HUVEC and coverage of stents, VCAM-1 decrease was correlated to better cellular migration [22]. Treatment with gevokizumab 1 mg/kg also reduced neointimal formation after carotid denudation, as measured by histomorphometry. The

![Figure 4](image1.png)

Fig. 4. Gevokizumab 1 mg/kg decreases total cell number and CD45-positive area in the neointimal tissue at day 28. (a) Total cell number quantification in the neointimal tissue on HPS-stained cross-sections of denuded carotid arteries (total number per section). $P = 0.049$, $^{**}P = 0.006$. Control group, $n = 8$; gevokizumab 1 mg/kg, $n = 10$; gevokizumab 10 mg/kg, $n = 10$; gevokizumab 50 mg/kg, $n = 10$; (b) CD45-labeled cross-sections of denuded carotid arteries from a control animal and a gevokizumab 1 mg/kg-treated animal (magnification ×10); (c) CD45-positive cell area quantification in the neointimal tissue of denuded carotid arteries (gevokizumab 1 mg/kg group ($n = 10$) versus controls ($n = 8$), $P = 0.23$).

![Figure 5](image2.png)

Fig. 5. Gevokizumab decreases the IL-1β-induced overexpression of endothelial adhesion molecules in vitro in cultured HUVECs. (a) E-selectin expression in HUVECs assessed by flow cytometry; (b) VCAM-1 expression in HUVECs assessed by flow cytometry. The percentages presented here report the ratios of the percent of cells significantly expressing the cell surface proteins under the different conditions relative to that of IL-1β stimulated cells, $N = 5$ experiments, triplicates for each experiment. Means and SEM are presented.
increased significantly the expression of both E-selectin and VCAM-1, when compared to controls (P < 0.01 and P < 0.05, respectively). The IgG2 control antibody alone did not exert any significant effect. Gevokizumab at a dose of 0.5 ng/mL decreased the IL-1β-stimulated expression of E-selectin (P < 0.01) but not VCAM-1 (P=NS), whereas higher doses (5 and 50 ng/mL) significantly decreased the IL-1β-induced expression of both E-selectin and VCAM-1 (P < 0.01). IL-1β: IL-1β at the dose of 50 U/mL; Gev 0.5: gevokizumab at the dose of 0.5 ng/mL; Gev 5: gevokizumab at the dose of 5 ng/mL; Gev 50: gevokizumab at the dose of 50 ng/mL. *P < 0.05, **P < 0.01, ***P < 0.001. Means and SEM are presented.
chemotherapy agent paclitaxel decreased neointima formation by more than 50% in a rat model of carotid denudation [23], but in contrast could delay reendothelialization, as corroborated by in vitro studies [24,25].

In the current study, we did not find any differences in the circulating levels of inflammatory cytokines (IL-1β, TNF-α, IL-6, data not shown). However, given that the current model involves a localized vascular lesion; its effect on circulating cytokines is expected to be modest. Therefore, the local tissue production of proinflammatory cytokines should be of greater interest than systemic release. With regards to RNA expression in carotid artery tissue, proinflammatory genes were increased in both the control and treated animals when compared with normal rats. These results support the validity of our model, as it corroborates the increased local inflammation occurring in denuded carotid arteries. We did not find any difference in the carotid tissue mRNA expression of proinflammatory genes between the treated and control animal groups; whether this could be attributed to subtle local effects remains to be determined. Of note, only the distal half of the denuded carotid arteries could be analyzed for gene expression. The timing of such analyses might also be crucial. We cannot exclude that only mild inflammatory processes were still active at 28 days. An earlier window for RNA and histological analyses could have also been of interest. Interestingly, in our in vitro experiments, the IL-1β-induced increase in HUVEC and rat SMC expression of adhesion molecules was inhibited by gevokizumab.

In our in vivo animal study, the most effective gevokizumab dose was 1 mg/kg, whereas non-significant effects were observed with 10 and 50 mg/kg doses. The effect of a high dose of gevokizumab on IL-1β signaling remains to be further investigated. Our findings with the 1 mg/kg dose are however in line with previous data showing better efficacy at 1 mg/kg than with 10 mg/kg for some markers of atherosclerosis in apoE knockout mice [18]. Furthermore, in our in vitro experiments, gevokizumab decreased the IL-1β-induced overexpression of endothelial adhesion molecules in HUVECs without an obvious dose–response relationship. This could reflect the complex impact of a cytokine modulating agent with differing effects at different doses. Whether dosages lower than 1 mg/kg could be of value in our model also remains to be determined.

4.1. Study limitations

Our study has limitations. First, potential early changes of inflammatory markers induced by gevokizumab may have been missed as blood samples were only taken at the time of sacrifice. Second, although the carotid denudation model is widely used in rats, the differences in severity of injury between animals should be considered. To address this issue, only one experienced operator performed all the experiments and was blinded to the randomized treatment assignment. Given that the 15 mm-long balloon introduced in the common carotid artery was bearing against the omohyoid muscle, the proximal region of the denuded vessel might have been the most injured one; this further highlights the observed benefit of gevokizumab on neointimal formation in this region.

4.2. Conclusion

A single infusion of the IL-1β modulator gevokizumab at 1 mg/kg leads to significant improvement of reendothelialization of denuded carotid arteries and reduction of neointimal formation, at least in part through its beneficial effects on endothelial cells.

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**Conflict of interest**

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2014.07.012.

**References**


