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Viral IL-10 Gene Transfer Decreases Inflammation and Cell Adhesion Molecule Expression in a Rat Model of Venous Thrombosis


Post-thrombotic inflammation probably contributes to chronic venous insufficiency, and little effective treatment exists. IL-10 is an anti-inflammatory cytokine that previously has been shown to decrease perithrombotic inflammation and thrombosis. We investigated in a rat model whether local expression of viral IL-10 (vIL-10) in a segment of vein that undergoes thrombosis would confer an anti-inflammatory effect and how this effect might be mediated. Rats underwent inferior vena cava isolation, cannulation, and instillation of saline or adenovirus encoding either β-galactosidase or vIL-10. Two days after transfection, thrombosis was induced, 2 days after this the rats underwent gadolinium (Gd)-enhanced magnetic resonance venography exam, and the vein segments were harvested. Tissue transfection was confirmed by either RT-PCR of vIL-10 or positive 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining. vIL-10 significantly decreased both leukocyte vein wall extravasation and area of Gd enhancement compared with those in controls, suggesting decreased inflammation. Immunohistochemistry demonstrated decreased endothelial border staining of P- and E-selectin, while ELISA of vein tissue homogenates revealed significantly decreased P- and E-selectin and ICAM-1 levels in the vIL-10 group compared with those in controls. Importantly, native cellular IL-10 was not significantly different between the groups. However, neither clot weight nor coagulation indexes, including tissue factor activity, tissue factor Ag, or von Willebrand factor levels, were significantly affected by local vIL-10 expression. These data suggest that local transfection of vIL-10 decreases venous thrombosis-associated inflammation and cell adhesion molecule expression, but does not directly affect local procoagulant activity.


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Deep vein thrombosis (DVT) sequelae commonly include pulmonary embolism and chronic venous insufficiency. While pulmonary embolism is often dramatic in presentation and immediately devastating in outcome, it is less common than venous insufficiency (1). Chronic venous insufficiency affects up to 30% of patients who suffer DVT and may be severely debilitating in up to 10% (1, 2). Unfractionated i.v. heparin effectively enhances natural clot lysis, but has little effect on perithrombotic venous inflammation (3). Limiting early perithrombotic inflammation may diminish later consequences, such as venous insufficiency and pain.

The molecular pathophysiology of DVT is beginning to be delineated. Thrombosis, like other physiologic processes, initiates a complex inflammatory mediator-procoagulant cascade that ultimately leads to vein wall damage (4–7). The process of thrombus resolution is similar to that of wound healing, with the nidus of injury being the clot, followed by leukocyte influx and eventually vein wall fibrosis. Mediators of this process include proinflammatory cytokines such as TNF-α and IL-1β (from the injured venous cells and local leukocytes) that serve to further activate leukocytes, stimulate chemotactic factor release, promote thrombosis, and increase cell adhesion molecule (CAM) expression (4, 6–9). Furthermore, thrombin acts as a proinflammatory mediator that not only amplifies the coagulation cascade, but also stimulates procoagulant changes in endothelial cells with concurrently increased CAM expression (3, 8, 10–12). The CAMs P- and E-selectin and ICAM-1 are critical for leukocyte tethering, firm adhesion, and emigration, respectively. Leukocyte emigration into the vein wall allows the release of cytotoxic inflammatory intermediates that cause vein damage (4, 6, 8, 9, 13–15). Prior work in our laboratory has clarified that TNF-α and P-selectin are central mediators of post-thrombotic vein wall inflammation and direct the magnitude of thrombus production (6, 13–15).

We have previously characterized an important role for IL-10 as an anti-inflammatory cytokine in the rat model of DVT. Specifically, neutralization of IL-10 resulted in increased local inflammation, while supplementation with human IL-10 (hIL-10) in a
dose- and time-dependent fashion decreased not only perithrombotic inflammation but also thrombus size (5). EBV encodes a homologue to rodent and human IL-10 that is designated viral IL-10 (vIL-10). vIL-10 has similar anti-inflammatory properties as hIL-10, but may not have the lymphocyte stimulatory properties associated with hIL-10 at high concentrations (16, 17). Other investigators have found that adenovirus encoding vIL-10 (Ad-vIL-10) transfection is associated with a significant reduction in alloantigen responsiveness, significantly increased cardiac allograft survival, decreased collagen-induced arthritis inflammation, and decreased systemic TNF and IL-1β release (18–21). Adenovirus-mediated gene transfer has the advantage of high efficiency, but is limited by transient gene expression and the host immune response against the viral infection (22, 23). Theoretically, local production of IL-10 via transfection has the advantage of minimizing confounding systemic effects, and its constant local expression may further enhance the anti-inflammatory effects compared with intermittent systemic infusions. In this study we show that Ad-vIL-10 transfection in a localized venous segment confers an anti-inflammatory effect during thrombosis and that this effect is probably mediated by down-regulation of CAM expression, but not through a reduction in proinflammatory mediator or procoagulant activity.

Materials and Methods

Adenoviral vectors

All vectors are E1 deleted. Ad-CMV encoding β-galactosidase (β-gal) or vIL-10 gene were under the control of the CMV immediate early promoter. Ad-RSV-β-gal was under the control of the RSV immediate early promoter. All vectors were obtained from the vector core of University of Michigan Medical Center and were produced as previously described (19).

Animal model, transfection, and tissue analysis

This rat model is a modification of a well-characterized method for reproducible stasis induced venous thrombosis (3, 5, 6, 13, 24). In brief, male Sprague Dawley rats, weighing 250–300 g, were anesthetized with an inhalation mixture of isoflurane (1–2%) and oxygen (100%) during the procedure. On day 1, aseptic laparotomy was performed, the inferior vena cava was isolated, and major side branches were ligated (Fig. 1). An 1.2-cm segment was dissected to allow temporary proximal and distal occlusion with microvascular clips. After occlusion, the inferior vena cava (IVC) was cannulated with a 30-gauge needle catheter, the blood was aspirated, and
the IVC was flushed with a dilute heparinized saline solution. After this, 0.15-mL instillation of saline, Ad-CMV-β-gal, Ad-RSV-β-gal or Ad-CMV-
Vil-10 (all 5 × 10^5 PFU/mL final concentration) was performed for 30 min. The vector solution or saline was aspirated, the clips were removed to re-establish blood flow, and the midline incision was closed. Two days after the transfaction, the rats were anesthetized in the same fashion, and a repeat laparotomy was performed. The IVC was then ligated below the renal veins for establishment of thrombosis. This method produces a clot in >95% of animals, and gradual re-establishment of flow usually occurs by dilation of posterior collaterals. Two days after thrombosis induction, the animals underwent gadolinium (Gd)-enhanced magnetic resonance imaging and were sacrificed. The thrombosed IVC segment was harvested below the renal veins and weighed (milligrams), and the weight was corrected to vein length (centimeters). The vein was then halved, and the proximal portion was placed in 10% buffered formalin for 24 h, followed by 70% ethanol for subsequent permanent section processing. The lower segment of the vein was snap-frozen in liquid N2 and kept at −70°C until homogenized.

**Gd-enhanced magnetic resonance venography (MRV) evaluation**

On the day of sacrifice, the rats were sedated with Telazol (20–40 mg/kg i.m.) and imaged with a magnetic resonance imaging system (Signa Horizon LX, Milwaukee, WI) using 8.1 software as previously described (24). Baseline time-of-flight imaging and T1 weighted imaging were performed followed by tail vein injection of 2 mL of Gd dimeglumine (0.2–0.4 mg/kg; Magnevist, Berlix, Wayne, NJ), and T1-weighted images were repeated. Gd is a heavy metal chelate that shortens the T1 relaxation time, prolonging the T1 relaxation time, followed by quenching of endogenous peroxidase of ethanol. The sections underwent Ag retrieval using 0.1% trypsin/deparaffinized with xylene followed by rehydration with graded dilutions of X-Gal solution (0.5 mg/ml X-Gal in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mL MgCl2, 1 mM spermidine, 0.02 Nonidet P-40, and 0.01% sodium deoxycholate in PBS). The sections were then fixed with 4% formaldehyde and counterstained with eosin.

**Histopathologic analysis and immunohistochemical staining**

Leukocyte infiltration into the vein wall was assessed as previously described (3, 5, 6, 13). In brief, leukocyte morphometric analysis was performed using a blinded fashion on hematoxylin- and eosin-stained sections. Each rat had three sections reviewed, and the section with the most complete circumference was chosen. Then, five high power fields (×1000) radially around the clot wall interface were assessed using standard criteria for neutrophils, monocytes (Mo), and lymphocytes. Random sections were taken from the proximal and middle portions of the whole IVC was reviewed, the most intense Gd-enhanced segment was chosen, and the area of maximum enhancement was measured in square millimeters in treated and controls rats.

**Immunohistochemical analysis was performed on the paraffin-processed tissue sections (10 μm) as previously described (6).** In brief, the slides were deparaffinized with xylene followed by rehydration with graded dilutions of ethanol. The sections underwent Ag retrieval using 0.1% trypsin/0.1% CaCl2 for 25 min, followed by quenching of endogenous peroxidase activity with methanol/3% H2O2 (50/50) for 15 min. On each slide after blocking, the sections were incubated with either control isotype specific Ab (goat IgG) or anti-E-selectin, anti-P-selectin (both 1 μg/ml), or anti-ICAM-1 (2 μg/ml; all from Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min. An anti-goat biotinylated Ab (1/1000) followed by avidin-peroxidase conjugate was added in sequence, and color development was performed with addition of diaminobenzidine substrate (ABC kit, Vector, Burlingame, CA). After color development, the slides were washed and counterstained with Mayer’s hematoxylin. Qualitative assessment of staining intensity was compared between the experimental and control groups.

5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining was performed on the control vector sections as described to confirm transfection (18). In brief, the rat vein sections were embedded with OCT (Miles Scientific, Naperville, IL) snap-frozen in liquid N2, sectioned at 10 μM, and fixed in 0.25% glutaraldehyde in PBS for 30 min. After rinsing in PBS for 30 min, the slides were incubated overnight at 37°C with the X-Gal solution (0.5 mg/mL X-Gal in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 1 mM spermidine, 0.02 Nonidet P-40, and 0.01% sodium deoxycholate in PBS). The sections were then fixed with 4% formaldehyde and counterstained with eosin.

The vein tissue (clot and wall) was thawed, placed in complete lysis buffer (Boehringer Mannheim, Indianapolis, IN), homogenized with a hand-held homogenizer, and sonicated for 10 s. The homogenate was centrifuged at 10,000 × g for 5 min, and the supernatant and sediment fraction were separated.

Quantification of inflammatory mediators and CAMs were all normalized to total protein in the sample. Total protein quantitation was performed using a modified Bradford assay according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). The protein concentration was determined in triplicate by reading absorbance at 595 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT). Standards were 0.5 log dilutions of the cytokines from 1 pg/ml to 100 ng/ml with a sensitivity of 50 pg/ml.

Analysis of the rat cytokine IL-1β and prostacyclin (estimated by 6-ke-to-PGF1α) levels were performed using a commercial ELISA and enzyme immunoassay, respectively, according to the manufacturer’s instructions with a sensitivity of 10 pg/ml (R&D Systems, Minneapolis, MN). The protein levels of tissue factor (TF) and von Willebrand factor (vWF) were analyzed using a human commercial ELISA with a sensitivity of ≥50 pg/ml or 10 milliunits/ml, respectively (American Diagnostics, Greenwich, CT).

F- and E-selectin and ICAM-1 levels were quantified in the cell homogenate using a direct ELISA method. In brief, 25 μL of the vein tissue homogenate or Ag-specific blocking peptide (for rat P- and E-selectin and ICAM-1) as standards (serial dilutions from 0.001–10 ng/ml; Santa Cruz Biotechnology) was mixed with coating buffer, added in duplicate, and incubated overnight at 0°C. The plates were washed three times with PBS and blocked with 2% BSA in PBS for 60 min at 37°C. After washing, 50 μL of Ab to P-selectin, E-selectin, or ICAM-1 (2 μg/ml; Santa Cruz Biotechnology) was added and incubated for 60 min at 37°C. After washing three times with PBS, a goat anti-rabbit peroxidase conjugate secondary Ab (1/200 dilution; Santa Cruz Biotechnology) was added and incubated for 45 min at 37°C. The plates were again washed, TMB was added for color development, and the reaction was quenched using 100 μL of 1 M H2PO4. The sensitivity was ≥10 pg/ml, and negligible background was found when testing the secondary Ab against the adsorbed Abs.

**Analysis of procoagulant activity**

An amidoletic assay was used for the determination of TF activity in the vein tissue homogenate (25). In brief, 50 μL of the vein tissue homogenate was added in duplicate to a microtiter plate followed by addition of the enzyme-substrate solution (1 U/ml Proplex T (Baxter-Allegiance Healthcare, McGaw Park, IL) and 200 μg/ml of S2222 (Helena Laboratories, Beaumont, TX) in medium 199 without phenol red (Life Technologies, Gaithersburg, MD) and with 3.24 mM CaCl2). This mixture was incubated for 30 min at 37°C in a humidified 5% CO2 incubator and then quenched with 3% HOAc. The plate was read at 410 nm. A standard of 0.01 nmol of labeled TF (American Diagnostics) was run under the same conditions, and the color development was arbitrarily set at 1 μM. The background of the homogenate without substrate enzyme mixture was subtracted from the sample. Negligible color development occurred in the absence of tissue homogenate.

**RT-PCR**

After removal of the vein tissue homogenate supernatant, the sediment fractions were pooled (n = 2–3) and placed in Trizol (Life Technologies, Gaithersburg, MD), which uses guanidinium-thiocyanate-phenol to isolate RNA. The RNA was extracted with chloroform/isooamyl alcohol and precipitated with 70% ethanol. The resulting RNA was quantified spectrophotometrically, and then 0.5–1 μg of RNA was reverse transcribed to cDNA by random primer reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was amplified using two specific primers for the VIL-10 gene as previously described (18, 26): 5′-ATGGAGGAGCGAAGGTTAGTG and 3′-TATGATTCATAGGCTTTTGATG.
GTC-3' (upstream) and 5'-ACTCTTGTTCTCACACGGCAG-3' (downstream). Forty PCR cycles yielded a 387-bp fragment using an automated thermocycler (RJ Research, Swedesboro, NJ) with cycles of 96°C for 15 s, 60°C for 30 s, and 72°C for 2 min. The cDNA-amplified product from a vIL-10 COS-transfected cell line served as a control (+, left column) and generated a 387-bp fragment. No bands were seen at this size in the saline or β-gal controls. In contrast, a distinct band at 387 bp was present in the vIL-10 group, confirming successful transfection. Similar levels of β-actin were seen in all groups.

**Results**

**Gene transfer and expression in the IVC**

The rat IVC was isolated, and transfection with the vector encoding β-gal or vIL-10 before thrombus induction was performed. X-Gal staining on Ad-LacZ control IVCs (n = 4) representing two separate transfection experiments demonstrated that about 20–30% of the inner vein wall circumference stained positive for β-gal expression (Fig. 2, A and B). RT-PCR analysis (pooled groups of three separate rat vein prepared segments; n = 3 separate transfection experiments) revealed that only the vIL-10-transfected group produced a 387-bp fragment consistent with vIL-10 (Fig. 2C). Neither the saline nor β-gal controls had any bands when RT-PCR was performed using the specific vIL-10 primers. However, all groups had similar intensity bands of the housekeeping gene β-actin, suggesting adequate starting cDNA.

**Perithrombotic inflammation is reduced by vIL-10 expression**

Assessment of inflammation was quantified by leukocyte vein wall emigration and Gd-enhanced MRV. Total subendothelial and medial leukocytes were all decreased with vIL-10 transfection compared with those in control saline and β-gal rats (n = 6–12; Fig. 3). Specifically, total leukocytes in the vein wall were significantly reduced by 36% (vIL-10, 33 ± 3; β-gal, 47 ± 4; saline, 56 ± 8), while neutrophils were reduced by 45% (vIL-10, 16 ± 3; β-gal, 27 ± 4; saline, 31 ± 6) compared with controls (p ≤ 0.05, by ANOVA, vIL-10 vs saline and β-gal; p < 0.05, by t test, vIL-10 vs β-gal).
vs. β-gal). Similarly, Mo extravasation was reduced by 27% (p < 0.05), and lymphocytes in the vein wall were rare in all groups.

To corroborate in vivo the reduction in vein wall leukocytes as well as physiologic effect, Gd-enhanced MRV was performed on the rats just before sacrifice (n = 6–12; Fig. 4). Coronal venous section analysis of the β-gal and saline control rats revealed 31 ± 4- and 31 ± 5-mm² average areas of perithrombotic Gd enhancement, respectively. In contrast, the rats transfected with vIL-10 had a decreased average coronal vein section area of perithrombotic enhancement of 17 ± 2 mm² (p = 0.056, by ANOVA, vIL-10 compared with saline and promoter controls; p < 0.05 by t test, vIL-10 compared with promoter control). Whereas the Gd enhancement was fairly diffuse in the control groups, the zone of enhancement was tighter and seemed to be mainly associated with the clot in the vIL-10 group. These data reflect a decreased amount of capillary leak and therefore decreased inflammation in the vIL-10-transfected group. No significant enhancement was observed before Gd injection by review of baseline scans (data not shown), and from previous work, nonclotted IVC has an average Gd enhancement of 1.8 mm² (24).

Vein tissue cytokine and PGI₂ analysis

ELISA analysis of the vein tissue homogenates showed a trend toward a decreased TNF-α level in the vIL-10 group compared with those in β-gal and saline controls, but this did not reach statistical significance (n = 2–4 groups of three pooled vein segments; Fig. 5A). In contrast, IL-1β was not significantly different
between the groups (n = 5–6; p > 0.05, by ANOVA). To test that endogenous rat IL-10 was not accounting for the differential effects observed, an ELISA for murine IL-10 that does not cross-react with vIL-10 was used. Indeed, no significant differences were found in local IL-10 levels, although a trend toward lower concentrations was found in the transfected animals (nearly 14-fold), while P-selectin was reduced 4-fold compared with levels in the control groups (p ≤ 0.05). ICAM-1 was also significantly reduced (3-fold) compared with the controls (p ≤ 0.05). However, this analysis does not take into account which cell types specifically have reduced CAM expression. Nonclotted vein tissue homogenates had negligible levels of CAMs (data not shown), reflecting low basal expression as well as the fact that no clot (or inflammatory nidus) was present.

**Thrombus size and procoagulant parameters are not affected by vIL-10 transfection**

Clot weight normalized to length was not reduced by vIL-10 expression (saline, 79 ± 8 mg/cm; β-gal, 65 ± 7 mg/cm; vIL-10, 65 ± 7 mg/cm; n = 10–15). No difference in vein-clot gross morphology was found between the groups. In accordance with the anatomical findings, functional TF activity, TF protein levels, and vWF levels were not significantly different in the vIL-10 group compared with the controls (n = 3–5; Fig. 8). Nonclotted vein wall analysis showed 2- to 3-fold less TF activity and Ag levels (data not shown). Thus, no direct difference in functional or biochemical measures of tissue coagulant activity was found with vIL-10 expression or adenovirus transfection.

**Discussion**

**IL-10 decreases perithrombotic inflammation**

In this animal model of venous thrombosis, we found that local expression of vIL-10 decreased inflammation. This finding correlates well with the results of our previous study in which hIL-10 given at time of thrombus induction significantly reduced inflammation (5). The absolute reduction in leukocytes (and the differential) was similar whether IL-10 was intermittently administered or constantly expressed, as presented here, suggesting a limit for the maximal anti-inflammatory effect. That no significant difference in rat cellular IL-10 levels was found between the groups suggests that the observed anti-inflammatory effect was specifically mediated by vIL-10 and not by endogenous IL-10 up-regulation. Indeed, the transfected control and vIL-10 rats had lower native IL-10 levels than saline controls. Although the local vIL-10 tissue levels were not directly determined, an unquestionable effect
was seen by two parameters of inflammation, vein wall leukocyte emigration and Gd-MRV. It is important to note that the aforementioned measures of inflammation correlate well with other measures of inflammation, including Evan’s blue dye extravasation (capillary leak) and myeloperoxidase activity (marker of polymorphonuclear cells (PMN)) (4–6, 13). Also congruent with our prior study was the finding that vIL-10 reduces PMN extravasation to the greatest extent of all the leukocytes. Neutrophils are the most prevalent early leukocyte after thrombosis and are probably a primary mediator of vein wall damage (5–7).

Other investigators have found similar anti-inflammatory efficacy with vIL-10 transfection in various experimental settings. For example, the collagen-induced arthritis rat model is similar to our model, in that a chronic inflammatory stimulus is present. With Ad-vIL-10 transfection, significantly decreased arthritic inflammation compared with that in controls was observed (20). Other animal models in which an inflammatory nidus is not present also support the thesis that local vIL-10 expression is anti-inflammatory. Specifically, in a murine cardiac allograft transplant model, retrovirally mediated vIL-10 transfection improved allograft survival by 3-fold (18). In a similar model using adenovirus-mediated vIL-10 transfer, vIL-10 expression accounted for a 2.5-fold increase in the duration of allograft survival (19). The authors also suggest that incorporating the vIL-10 gene may decrease the host immune response to the adenovirus itself, a significant problem with this method of gene transfer (22, 23, 27–29). The anti-inflammatory effect of vIL-10 in these studies was diminished T cell responsiveness and cytotoxicity. We did not specifically look at these T cell parameters, but it is likely that this effect plays only a small role in the diminished inflammation that was observed because lymphocytic infiltration is quantitatively a small component of the total vein wall leukocytes. We also did not find any evidence of increased inflammation in the Ad-β-gal control group, perhaps because a significant inflammatory stimulus was already present and “hid” any additional inflammatory effect of the viral transfection.

FIGURE 6. Composite photomicrographs of immunohistochemistry and hematoxylin- and eosin-stained stains of rat vein segments. W, vein wall. A, Vein wall section stained for E-selectin in a saline control rat (×1000). Note endothelial lining staining of modest intensity (black arrows) with a few stained medial leukocytes. B, Vein wall section stained for E-selectin in a β-gal control rat (×1000). Most staining is on the endothelial border, as shown by black arrows. C, Vein wall section stained for E-selectin in the vIL-10 group (×1000). Minimal luminal staining is seen compared with that in A and B (as demarcated by black arrow). D, A vein wall section stained for P-selectin in a saline control rat is shown (×1000). Note the extensive fluffy enhancement luminally (black arrows; E). A vein wall section stained for P-selectin in a β-gal control is shown (×1000). The staining intensity was similar to that in D. F, Vein wall section stained for P-selectin in a vIL-10-transfected rat (×1000). Minimal staining was found (black arrow, G). Hematoxylin and eosin staining of a saline control rat vein section is shown (×400), illustrating a heavy infiltration of leukocytes in the subendothelial (white arrowhead) and medial areas (white arrows). The endothelial border is marked by a black arrow. H1, Hematoxylin and eosin staining (×1000) of a β-gal control, illustrating PMN infiltration of subendothelial region (white arrow) and media (white arrowhead). H2, Hematoxylin and eosin staining (×1000) of a β-gal control, illustrating subendothelial Mo extravasation. I, Hematoxylin and eosin staining of a vIL-10-transfected rat vein section (×400), showing a few scattered leukocytes (white arrows) in the media and a relatively intact endothelial layer (black arrows).
FIGURE 7. Quantitative representation of CAM levels in vein tissue homogenates. A. The E-selectin level (picograms per milligrams of protein) is significantly less than those in the β-gal and saline controls. B. The P-selectin level (nanograms per milligram of protein) is significantly less than those in saline and β-gal controls. C. The ICAM-1 level (picograms per mg protein) is significantly less than those in saline and β-gal controls. For all graphs: * p < 0.05, by ANOVA. Note the relative absolute differences between the CAM concentrations; P-selectin is greatest, followed by E-selectin, and ICAM-1 is the least.

FIGURE 8. Graphical representation of vein tissue coagulant markers. No difference was found between the groups in any of these parameters (p > 0.05, by ANOVA). A. Vein tissue homogenate procoagulant activity as reflective of functional TF activity in milliunits per milligrams of protein. B. Tissue factor Ag levels in picograms per milligrams of protein. C. vWF levels in vein tissue homogenates in milliunits per milligrams of protein.
Also, at 4 days after transfection an adoptive immune response is not expected.

**Viral IL-10 decreases CAM up-regulation**

Cell adhesion molecule up-regulation occurs in the setting of multiple biologic events, such as wound healing, atherosclerosis, and cancer. Indeed, the regulation of CAMs is very complex and controlled in part by the balance of pro- and anti-inflammatory cytokines (4, 8, 9). However, while the specific stimuli for increased CAM expression are fairly well defined, inhibitors of expression are less well known. In the setting of thrombosis, increased CAM expression is seen at the onset of thrombus induction and facilitates stabilization of the fibrin-platelet leukocyte complex as well as allows the proper recruitment of leukocytes for clearance and resolution of the inflammatory process (8, 9, 11, 12). Most experimental data of CAM kinetics have been made in flow situations, whereas it is less clear what occurs in a blood stasis model. Furthermore, the kinetics and response of the CAMs are different when comparing in vitro to in vivo situations. For example, P-selectin is rapidly mobilized after a stimulus such as thrombin, whereas E-selectin expression increases several hours later (4, 8). However, in vivo, persistent up-regulation of both P and E selectin may occur, depending on the inflammatory stimulus (30, 31). In the setting of venous thrombosis, an inflammatory stimulus (clot) is present that modulates CAM up-regulation, with P-selectin present in greatest magnitude.

Although we have only shown an association, our data strongly argue that vIL-10 mediates its anti-inflammatory action through down-regulation of CAM expression. In support of this contention, other investigators have found that IL-10 mediates some of its anti-inflammatory activity through decreased CAM expression. Specifically, in an acute lung injury model, IL-10, but not IL-4, significantly decreased lung injury (hemorrhage and myeloperoxidase activity), and this was associated with decreased ICAM-1 levels, probably due to concurrently decreased TNF-α levels (32). An IL-10 knockout mouse model further enforces the close relationship between IL-10 and selectin regulation. After a low grade septic insult, the IL-10 knockout animals had markedly increased leukocyte adhesion in mesenteric venules and increased capillary leak and myeloperoxidase activity compared with wild-type animals. Importantly, when anti-P- and anti-E-selectin Abs were given, reduction of the leukocyte adherence was observed, suggesting unbalanced CAM up-regulation that was not seen in the wild-type mice (33). Even more convincingly, administration of either Ab to P-selectin or a specific soluble P- and E-selectin antagonist, P-selectin glycoprotein ligand-1, significantly reduced inflammation and thrombus, as measured biochemically, anatomically, and by Gd-MRV in a non-human primate model of venous thrombosis (14, 15). Both of these models involved experimental analysis beyond 2 days, suggesting that early inhibition of the selectin-mediated events may confer later anti-inflammatory effect and preserve vein wall integrity. Other models support the interrelation between IL-10 and CAMs. IL-10 similarly blunted inflammation secondary to myocardial ischemia-reperfusion through an ICAM-1 mechanism (34), while in a mouse septic shock model, IL-10 reduced liver injury and mortality through an ICAM-1 and VCAM mechanism (35).

In vitro cell culture systems have yielded some conflicting insights into the modulatory action of IL-10 on CAM expression. In an LPS-stimulated glial cell line, IL-10 significantly reduced the expression of ICAM-1 but not its message level (36). Similarly, an IL-1-stimulated human umbilical vascular endothelial cell line had ICAM-1 and VCAM expression significantly reduced by IL-10 in a dose-dependent manner and correlated with diminished Mo and lymphocyte adhesion (37). In contrast, IL-10 administration in a human microvascular endothelial cell line actually stimulated E-selectin expression (38). These data suggest possibly a direct effect of IL-10 on endothelial CAM expression. However, experiments with human umbilical vascular endothelial cells in our laboratory have failed to show any direct modulating effect of IL-10 (over a wide concentration range) on E-selectin, ICAM-1, or procoagulant activity (unpublished observations). These results underscore the important functional heterogeneity of endothelial cells as well as the local environmental influences (4, 7, 8). As such, we believe that vIL-10 probably works indirectly on CAM expression.

The observation that P-selectin localizes rapidly and specifically to the endothelial border after thrombin stimulation underlies the importance of its role in the initial clot formation and subsequent inflammation (12, 39). Our immunohistochemical data suggest decreased luminal expression of the selectins in the vIL-10-transfected animals, and we hypothesize that luminal expression of the selectins is most important for leukocyte emigration. IL-10 may decrease the CAM expression on other cell types besides endothelial cells, and our results with the homogenate ELISA were not able to differentiate specific cell type levels. For example, IL-10 exerts an inhibitory effect on Mo ICAM-1 expression (40) that, again, may confer an anti-inflammatory effect by counterligand inhibition.

**Viral IL-10 and its relationship to other inflammatory mediators**

IL-10 is a pleiotropic cytokine derived primarily from Mo and T lymphocytes that has many other anti-inflammatory actions besides CAM modulation and may also account for the decreased inflammation observed in these studies. Importantly, IL-10 inhibits Mo Ag-presenting capacity and IL-1β and TNF-α production, as well as increases IL-1R antagonist levels and lymphocyte IL-4 production (16, 17, 41). Furthermore, the neutrophils in the vein wall may have a diminished release of reactive oxygen intermediates, while Mo nitric oxide and chemotactic chemokines are reduced by IL-10 (42, 43). TNF-α is an important proximal stimulus for CAM up-regulation (4, 8, 9), and although we only found a trend in decreased TNF-α in the vIL-10-transfected animals, this may be physiologically important. Two hypotheses may account for the nonsignificant differences found in TNF-α. First, the clot environment is more static than that of other inflammation models; thus, the TNF-α Ag may not be cleared as readily (falsely elevated local levels). Secondly, the measured TNF-α levels may not correlate with functional activity. Alternatively, other inflammatory mediators, such as CSa, IL-6, and IFN-γ, may also be down-regulated by IL-10 and indirectly account for the decreased CAM levels (16, 17).

Interestingly, PGI₂ was significantly less in β-gal compared with vIL-10 animals. PGI₂ is a vasodilatory and antithrombotic PG that is adaptively up-regulated in chronic thrombosis (44). IL-1β levels correlated with PGI₂ levels (although not significantly different between the groups), and it is well documented that IL-1β is a proximal stimulus for PGI₂ production (45, 46). Thus, it seems that vIL-10 expression preserves and may stimulate the antithrombotic PGI₂ response, and that adenovirus transfection inhibits this compensatory response to chronic thrombosis compared with that in saline controls. Furthermore, our in vivo data are supported by in vitro experiments showing that IL-1β-stimulated PGI₂ production is dissociated from vWF production (47). Additionally, PGI₂ has anti-leukocyte adhesive properties (48) that may have contributed to the observed decrease in vein wall leukocytes in addition to the decreased CAM expression.
Viral IL-10 does not affect tissue coagulation mechanisms

The interrelationship between CAM up-regulation and thrombosis is well defined (7–9, 11–15). P-selectin may be particularly important, as it directly links platelets, leukocytes, and endothelial cells to propagate thrombus. Our prior experiments support the interrelation between inflammation and thrombus production, as exogenous IL-10 decreased clot weight compared with control values (5). In the current study, however, expression of vIL-10 did not inhibit thrombus production as assessed by clot weight, nor did it affect any of the clotting parameters measured. This might relate to a difference in receptor binding constants between vIL-10 and endogenous cellular IL-10 despite the high sequence homology between these molecules (41). IL-10 decreases Mo TF activity (49), and thus, systemic administration may more fully deactivate this than when vIL-10 is localized at the thrombus site. This hypothesis is also supported by the fact that only IL-10 given at the time of thrombus induction was an effective antithrombotic agent (5). It is more likely that adenovirus transfection itself stimulated the procoagulant state that was not overcome by vIL-10. Adenovirus transfection (with or without β-gal expression) in normal rabbit arteries is associated with a significant inflammatory response, in-transfection (with or without 

Local expression of vIL-10 effectively reduces early vein wall inflammation, probably by indirect down-regulation of P- and E-selectin and ICAM-1 expression. The important question remains, however, as to whether this reduces late vein wall damage or affects the development of chronic venous insufficiency. Additional experiments to better delineate the differences between vIL10 and rIL10 are planned by systemic administration, which might abrogate differences in gene expression variability. Using specific CAM knockout mice would also clarify the actions of vIL10 in this system. Although our methodology of segmental transfection of an anti-inflammatory mediator was used here as a tool to delineate its effect and mechanisms, clinical applicability is foreseeable. However, before clinical use of such an anti-inflammatory cytokine, further physiologic experimental measures, such as distal femoral venous pressure, ultrasound analysis of clot resolution and vein wall thickness, and vein wall analysis for fibrosis, should be undertaken. Furthermore, longer duration transfection studies to determine the durability of the effect of anti-inflammatory cytokines would be important before any clinical use. Given the unrmitting nature of chronic venous insufficiency (CVI) and the increased likelihood of rethrombosis in an anatomically diseased segment, local gene-specific anti-inflammatory or anti-coagulant mediators are appealing. The complications from long term anticoagulation are significant (1), and selectively preventing vein wall rethrombosis and/or inflammatory damage without systemic anticoagulation is important and worth further investigation.

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References

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