Tumor necrosis factor inhibitors as novel therapeutic tools for vascular remodeling diseases

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Lambert CM, Roy M, Meloche J, Robitaille GA, Agharazii M, Richard DE, Bonnet S. Tumor necrosis factor inhibitors as novel therapeutic tools for vascular remodeling diseases. Am J Physiol Heart Circ Physiol 299: H995–H1001, 2010. First published August 13, 2010; doi:10.1152/ajpheart.00562.2010.—Vascular remodeling diseases (VRDs) are characterized by enhanced inflammation and proliferative and apoptosis-resistant vascular smooth muscle cells (VSMCs). The sustainability of this phenotype has been attributed in part to the activation of the transcription factor hypoxia-inducible factor-1 (HIF-1). There is evidence that circulating cytokines can act as HIF-1 activators in a variety of tissues, including VSMCs. Increased circulating tumor necrosis factor (TNF) levels have been associated with vascular diseases, but the mechanisms involved remain unknown. We hypothesized that increased circulating levels of TNF promote VRDs by the activation of HIF-1, resulting in VSMC proliferation and resistance to apoptosis. Circulating TNF levels were significantly increased in patients with vascular diseases (n = 19) compared with healthy donors (n = 15). Using human carotid artery smooth muscle cells (CASMCS), we demonstrated that TNF (100 ng/ml) activates HIF-1 (HIF-1α expression), leading to increased CASMC proliferation (Ki-67 and PCNA staining) and resistance to mitochondrial-dependent apoptosis [tetramethylrhodamine methyl ester perchlorate (TMRM), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), annexin-V staining]. In vivo, TNF inhibition using polyethylene glycol coupled with TNF membrane receptor 1 (PEGsTNFR1), a soluble TNF receptor inhibiting potential hyperpolarization and resistance to apoptosis (23, 28).

Several lines of evidence indicate that HIF-1 can be activated by cytokines and growth factors under normoxic conditions (3, 5, 12, 18, 23). TNF is a cytokine for which the circulating level has been correlated to higher cardiovascular risks (29, 34). Moreover, TNF has been shown to induce HIF-1 activation in various cell lines (4, 11, 16, 35). Taking together, these findings suggest a potential link between circulating TNF and HIF-1 activation in VRDs.

HIF-1 is a heterodimeric transcription factor consisting of α- and β-subunits and usually activated by low oxygen concentrations. In normoxic conditions, the HIF-1α subunit is rapidly degraded. Under hypoxia, HIF-1α accumulates and allows the nuclear translocation of the heterodimer and its binding to targeted promoters (13). HIF-1 regulates the expression of a variety of genes involved in angiogenesis, metabolism, proliferation, and survival (5, 17, 23, 37). Recently, we described that Akt-dependent activation of HIF-1 accounts for the metabolic shift from glucose oxidation to complete glycolysis seen in proliferative VSMCs. Indeed, by increasing hexokinase 2 (HXK2), HIF-1 activation leads to mitochondria membrane potential hyperpolarization and resistance to apoptosis (23, 28).

Several lines of evidence point to a role for cytokines in the etiology of VRDs (1, 8, 9, 27, 32). For example, several studies have shown that patients with higher levels of circulating inflammatory markers, including the proinflammatory cytokine TNF, have greater cardiovascular risks (29, 34). Moreover, TNF has been shown to induce HIF-1 activation in various cell lines (4, 11, 16, 35). Taking together, these findings suggest a potential link between circulating TNF and HIF-1 activation in VRDs.
MATERIALS AND METHODS

All experiments were performed with the approval of the Université Laval’s Ethic and Biosafety Committee and the Centre Hospitalier Universitaire de Québec’s Ethic’s Committee. The investigation conforms with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and with the principles outlined in the Declaration of Helsinki.

TNF measurement. The TNF level was measured by ELISA as described in the manufacturer’s protocol (R&D Systems, Minneapolis, MN). TNF was measured in serum from 19 patients with cardiovascular diseases (as defined by coronary artery disease, left ventricular hypertrophy, and peripheral artery disease) and 15 control patients (Table 1). Blood samples have been taken after one month without infection. All patients provided informed, written consent. TNF was also measured by ELISA as described in the manufacturer’s protocol (R&D Systems) in plasma from control and carotid injured rats (n = 5 per group).

Cell culture. hCASMCs isolated from a healthy donor (organ transplant patient) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% of an antibiotic-antimycotic mixture (GIBCO 15240, Invitrogen) and maintained in a humid atmosphere at 37°C with 5% CO₂-95% room air. Cells were used between passages 3 and 8.

Drugs and peptides. hCASMCs were treated with recombinant human TNF (100 ng/ml, Feldan Bio) and phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002 (10 µM, EMD Chemicals; Gibbstown, NJ) for 48 h in DMEM with 10% of FBS at 37°C. Cells were treated at 48 h posttransfection. Rats were treated with PEGsTNFR1 at 2 mg/kg (Amgen). PEGsTNFR1 is a soluble TNF receptor that inhibits circulating TNF and blocks its binding on membrane receptors.

Transfection. hCASMCs were transfected by CaPO₄ precipitation with 20 nM small interfering RNA (siRNA) oligonucleotides in DMEM with 10% of FBS. The transfection agent is a solution of 5% CaCl₂ (2.5 M) and 50% of HBS (2X). A ratio of 100 µl of transfection solution per milliliter of medium is done. At 24 h posttransfection, the cell medium was changed, and at 48 h posttransfection, the cells were treated as indicated. All siRNAs were obtained from Applied Biosystems, and the specific sequences used were as follows: human HIF-1α (accession no. NM_001530; sense, 5’-AGGACAAGUCACAAAGGAAU-3’). As a control oligonucleotide, Silencer Negative Control No. 2 siRNA was used.

Immunoblotting. Immunoblotting was performed with HIF-1α (1:1,500, antiserum raised in rabbits immunized against the last 20 amino acids of the COOH-terminal human protein) (33), Akt (1:1,000, Cell Signaling), phospho-Akt (1:1,000, Cell Signaling), HXK2 (1:1,000, Cell Signaling), TNFR1 (1:1,000, Abcam), TNFR2 (1:1,000, Abcam), and actin (1:300, Santa Cruz). Protein (25 µg) was loaded. Expression was normalized to actin to correct for loading differences.

Confocal microscopy. The mitochondrial membrane potential was determined using 10 nM tetramethylrhodamine methyl ester perchlorate (TMRR, Invitrogen). Nuclei were stained using 50 nM Hoechst 33342 (Invitrogen). Cell imaging was performed using a FV1000 confocal microscope equipped with a live cell apparatus (Olympus, Center Valley, PA).

Immunofluorescence. hCASMCs were fixed with 1% paraformaldehyde and permeabilized with 0.2% Triton X-100. Apoptag apoptosis detection kit (TUNEL, Serologicals, Norcross, GA) and annexin V (Clonetech) were used to measure apoptosis after starvation. The percentages of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and annexin-V positive cells were calculated. Ki-67 (1:200, Millipore) and PCNA (1:400, Dako) were used to measure proliferation by calculating the percentage of Ki-67 and PCNA positive cells. Primary antibody detection was performed using Alexa Fluor 488 or 594 nm secondary antibodies (Invitrogen).

Rats common carotid arteries were fixed with 4% paraformaldehyde. Immunofluorescence was performed on 5-µm carotid slices. Antibodies to HIF-1α (1:200), Ki-67 (1:200), TNF-α (1:100, Millipore), TNFR2 (1:200, Abcam), von Willebrand factor (1:250), and α-smooth muscle actin (1:400, Sigma) were used. An analysis was done in five rats per group (n = ~200 VSMCs per rat; and n = ~100 in neointima and ~100 in media). Five pictures have been taken per rat to have the entire arterial wall.

Carotid artery balloon injury model. Male Sprague-Dawley rats (350 g) were used. Under anesthesia, a neck incision was made. A 20-mm section of the right common carotid artery was isolated and temporarily occluded to prevent retrograde blood loss. After proximal arteriotomy, a 2-Fr Fogarty embolectomy catheter was introduced to perform an anastomode balloon inflation along a 15-mm segment of the common carotid artery. The lumen was then flushed with heparinized saline, the arteriotomy was closed with 9-0 prolene sutures, and the perfusion was restored. Subcutaneous injections of PEGsTNFR1 (2 mg/kg) were performed 3 days after surgery, 3 times per wk for 14 days. Hematoxylin and eosin staining was performed on carotid slides. Media and neointima thickness was measured 10 and 14 days postinjury (5 slides per rat/5 rats were studied). Experiments were performed with the approval of Université Laval’s Animal Ethic Committee.

Statistics. Values are expressed as means ± SE. ANOVA was performed with Newman-Keuls test for post hoc analysis. P < 0.05 was considered significant (GraphPad Prism, version 5.0b).

RESULTS

TNF levels are increased in serum of patients with VRDs and carotid injured rats. TNF was measured by ELISA from 15 control patients and 19 VRD patients. As seen in Fig. 1A, TNF levels were significantly increased in patients with VRDs compared control patients (6.3 ± 1.8 to 11.7 ± 3.1 pg/ml, P < 0.001). Similarly TNF levels were also increased in rats following injury. As seen in Fig. 1B, TNF levels increased with the development of the disease and reach a maximum at 10 days postinjury (n = 5 per group, P < 0.05). After 10 days, a decrease in TNF levels was observed (although it remains significantly higher than the levels seen in control). Taking together, these findings show that TNF levels increased during the development of diseases.

HIF-1 activation by TNF increases HXK2 expression, leading to hyperpolarization of mitochondrial membrane, decreasing apoptosis, and enhancing hCASMC proliferation. The treatment of hCASMCs with TNF (100 ng/ml for 48 h)
promoted the upregulation of HIF-1α by 1.5 ± 0.2-fold over untreated cells (Fig. 2A) (n = 4; P < 0.05). The transfection of TNF-treated hCASMCs with a HIF-1α siRNA decreased HIF-1α protein expression by ~70 ± 5% compared with TNF-treated cells transfected with a control siRNA (n = 3; P < 0.05), HIF-1 activation leads to the upregulation of HXK2. As seen in Fig. 2B, HXK2 protein expression was increased (2 ± 0.5-fold) by TNF. Targeting HIF-1α with a specific siRNA significantly reduced HXK2 protein levels by 75 ± 30% in TNF-treated hCASMCs compared with the control siRNA (n = 3; P < 0.05). Interestingly, inhibiting the PI3K/Akt axis with LY-294002 decreases HXK2 expression to a level similar to the one seen by HIF-1 inhibition. The fact that PI3K/Akt inhibition mimicked HIF-1 inhibition confirms our previous finding that HIF-1 is PI3K/Akt regulated (23).

As expected, HXK2 upregulation by TNF leads to the hyperpolarization of the mitochondrial membrane potential measured by TMRM staining (n = 500; P < 0.001). Once again, this hyperpolarization was blocked by both LY-294002 and HIF-1 siRNA (Fig. 2C). Finally, mitochondrial hyperpolarization by TNF resulted in resistance to serum deprivation induced apoptosis, measured by both annexin V and TUNEL staining (n = 250; P < 0.01) (Fig. 2D). As predicted, both HIF-1 and PI3K/Akt inhibition blocked apoptosis resistance.

We previously published that the activation of Akt (6) and HIF-1 (23) resulted in a significant increase in VSMC proliferation. This finding was indeed confirmed in our TNF-treated hCASMCs by Ki-67 and PCNA staining, showing that TNF promoted hCASMC proliferation by 11 ± 2% (n = 250; P < 0.05) (Fig. 2E), whereas both HIF-1 and Akt inhibition blocked increased hCASMC proliferation by TNF.

In conclusion TNF promotes HIF-1 activation, resulting in the development of pro-proliferative and apoptotic resistant state in human CASMCs.

**TNF inhibition prevents carotid artery wall remodeling.** As seen in Fig. 3, both media and neointima wall thicknesses significantly increase between 10 and 14 days postinjury in saline-treated injured rat carotids (saline carotids) compared with sham-operated rats (sham carotids). This effect is associated with an increase in both circulating TNF levels (Fig. 1B) and VSMC-produced TNF (supplemental Fig. 1A; note: supplemental material may be found posted with the online version of this article). Both media and neointima wall thickness were significantly decreased in rats with injured carotids treated for 14 days with PEGsTNFR1 (PEGsTNFR1 carotids) (2 mg/kg, 3 times per wk, n = 5 per group, P < 0.05). Immunofluorescent analysis of carotid slides with an anti-α-smooth muscle actin antibody (in green) indicates that smooth muscle cells are present in the entire vascular wall and that the neointima of injured carotids and contribute to their thickening. Triple staining with HIF-1α (in red), α-smooth muscle actin (green), and DAPI (nucleus blue), followed by an increase in VSMC proliferation within the neointima (14 days). As expected, HIF-1 activation in saline-treated animals is first associated with a significant increase in CASMC proliferation within media (10 days) with increased Ki-67 (in red), α-smooth muscle actin (green), and DAPI (nucleus blue), followed by an increase in VSMC proliferation within the neointima (14 days). These effects were reversed by PEGsTNFR1 treatments (5 rats per group; n = ~200 per rat, n = ~100 in neointima, and n = ~100 in media) (P < 0.05 and P < 0.001). Western blot analysis of HIF-1α confirmed our results (supplemental Fig. 2). 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potential by promoting HXK2 translocation to mitochondria, suppressing hCASMC ability to undergo apoptosis and promoting hCASMC proliferation (23). These phenomena are reversed by HIF-1 inhibition. In vivo, we demonstrate that systemic TNF inhibition using the TNF inhibitor PEGsTNFR1 prevented carotid artery postinjury remodeling in rats by decreasing rat CASMC proliferation.

Our findings confirmed the implication of TNF and HIF-1 in VRDs. We described that elevated TNF levels activate HIF-1, promoting VSMC proliferation and resistance to apoptosis, confirming the importance of HIF-1 in VRDs (5, 36). HIF-1 activation in VRDs has been attributed to the PI3K/Akt/GSK3β axis and that the inhibition of this axis resulted in the inhibition of HIF-1 activation. In the present study, we clearly showed that similarly to the TNF-dependent HIF-1 inhibition, PI3K/Akt inhibitors decrease proliferation and promote apoptosis, suggesting that in our model TNF promotes HIF-1 through an PI3K/Akt-dependent mechanism. Western blot analysis supports this hypothesis (supplemental Fig. 3). Nonetheless, because of the numerous mediators involved in the TNF pathway, it is likely that in addition to the Akt pathway, other mechanisms might explain in part our results. Indeed, TNF effects have been shown to be mediated by Jak/STAT and NF-κB (15, 38, 40), all of which can also activate HIF-1 (39) and promote apoptosis resistance (25) and cell proliferation (10, 20, 30). These different pathways are activated through TNF membrane receptors (TNFR1 and -R2), both present on CASMCs in vitro and in vivo, as shown by preliminary data (supplemental Fig. 1, A, C, and D). TNFR1 activation has been associated with the activation of pro-apoptotic pathways, whereas TNFR2 with pro-proliferative ones. In our study, we show a trend in the upregulation of TNFR2, consistent with CASMC proliferation and resistance to apoptosis (38). Therefore, it is believed that whatever signaling pathway is triggered

Fig. 2. Hypoxia-inducible factor (HIF)-1 activation by TNF increases hexokinase-2 (HXK2) expression, leading to hyperpolarization of mitochondrial membrane, decreasing apoptosis, and enhancing human carotid artery smooth muscle cell (hCASMC) proliferation. A: hCASMCs stimulated with TNF-α (100 ng/ml) for 48 h had elevated expression of HIF-1α (1.5-fold) by Western blot analysis. Transfection with HIF-1α silencer RNA decreased about 70% of its expression (n = 3). B: HXK2 expression is increased in TNF-stimulated cells (2-fold). HIF-1α inhibition by small interfering RNA (siRNA) or treatment with LY-294002 decreased HXK2 expression in TNF-α-stimulated cells (75%) as shown by Western blot analysis (n = 3). C: TNF-α-stimulated hCASMCs have hyperpolarized ΔΨm vs. control cells. HIF-1α inhibition or treatment with the phosphatidylinositol 3-kinase inhibitor LY-294002 induced mitochondrial depolarization (n = −500). TMRM, tetramethylrhodamine methyl ester perchlorate; FU, fluorescence units. D: in TNF-treated cells, HIF-1α inhibition or treatment with LY-294002 promoted apoptosis, measured by terminal deoxynucleotidetransferase-mediated dUTP nick end labeling (TUNEL) and annexin-V staining (n = −250). E: HIF-1α inhibition or treatment with LY-294002 in TNF-stimulated cells decreased proliferation measured by Ki-67 and PCNA staining (n = −250). *P < 0.05; **P < 0.01; ***P < 0.001.
by TNF, it will result in HIF-1 activation in VSMCs and will promote cell proliferation and resistance to apoptosis. This indeed reinforced the importance of TNF in vascular remodeling and clearly present TNF as a very attractive therapeutic target for vascular diseases.

There is growing evidence that TNF is involved in cardiovascular diseases. For example, elevated circulating TNF levels have been associated with a greater risk of developing vascular diseases (22). Mice lacking TNF have attenuated intimal hyperplasia following carotid injury (40), whereas an overexpression of TNF in mice worsens pulmonary hypertension, an accepted VRD (14). Furthermore, in a recent case report, the TNF antibody infliximab reduced pulmonary pressures and improved the quality of life in a patient with pulmonary arterial hypertension secondary to advanced scleroderma (2). In addition to the inhibition of HIF-1 pathway that we propose, improved vascular functions by TNF inhibition in injury/angioplasty model is also likely associated to improve endothelial function. Indeed, several studies showed that TNF inhibition accelerates functional endothelial regrowth (19, 21).

In agreement with these studies, in our model we have preliminary evidence that in PEGsTNFR1-treated rats, there are more endothelial cells (shown by a greater von Willebrand factor positive cells). Although, more experiments are needed to evaluate whether this observation is due to endothelial regrowth and whether the endothelium is functional (21, 26) (supplemental Fig. 4). Thus, by inhibiting the HIF-1 pathway in VSMCs and by promoting endothelial regrowth, TNF inhibitors might represent an attractive and novel way of treating vascular diseases.

The mechanisms resulting in elevated TNF levels remain elusive and are of great clinical interest. We previously published...
lished that the nuclear factor of activated T cells (NFAT) is increased in patients with VRDs (6, 7). Since TNF expression is driven in part by NFAT, we speculated that the activation of NFAT may lead to increased levels of TNF in VRD patients. This hypothesis is in agreement with our previously published findings showing that NFAT inhibition reverses VRDs (6, 7) by decreasing Akt pathway, VSMC proliferation, and resistance to apoptosis.

In conclusion, the present study offers a strong and direct translational potential. It provides a rationale for clinical translation of TNF inhibitors in vascular diseases since they are already in clinical use for autoimmune diseases. The greatest benefit may be in VRDs associated with autoimmune diseases, particularly those with elevated serum levels of TNF; this can be easily measured as shown by our data and thus “targeted” clinical trials in patients with high serum TNF levels are possible. We propose that the development of slow-release TNF inhibitor-coated stents could be a novel therapeutic strategy to prevent restenosis.

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DISCLOSURES
S. Bonnet signed an MTA with Amgen, Inc., to get PEG-TNFR1. PEG-TNFR1 was provided by Amgen, although the authors did not receive any funding from Amgen.

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