

Transthyretin Proteins Regulate Angiogenesis by Conferring Different Molecular Identities to Endothelial Cells*

Received for publication, March 18, 2013, and in revised form, September 12, 2013. Published, JBC Papers in Press, September 12, 2013, DOI 10.1074/jbc.M113.469858

Raquel J. Nunes^{‡1}, Paula de Oliveira[‡], Ana Lages[§], Jörg D. Becker[¶], Paulo Marcelino^{||}, Eduardo Barroso^{**}, Rui Perdigoto^{**}, Jeffery W. Kelly^{††§§}, Alexandre Quintas[§], and Susana Constantino Rosa Santos^{‡2}

From the [‡]Angiogenesis Unit, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, 1649-028 Lisbon, Portugal, the [§]Centro de Investigação Interdisciplinar Egas Moniz, Instituto Superior das Ciências da Saúde Egas Moniz, Campus Universitário, Monte da Caparica 2829-511 Caparica, Portugal, the [¶]Instituto Gulbenkian de Ciência, 2781-901 Oeiras, Portugal, the ^{||}Centro de Estudos de Doenças Crônicas, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, 1169-056 Lisbon, Portugal, the ^{**}Liver Transplant Center, Curry Cabral Hospital, 1069-166 Lisbon, Portugal, the ^{††}Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California 92037, and the ^{§§}Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

Background: The biological effects of transthyretin proteins on vasculature remain unknown.

Results: V30M transthyretin tetramer modulates endothelial global gene expression, down-regulating pro-angiogenic genes, inducing apoptosis and inhibiting migration.

Conclusion: Transthyretin proteins regulate angiogenesis by conferring different molecular identities to endothelial cells.

Significance: This work has critical implications in the prevention of early hepatic artery thrombosis in familial amyloidotic polyneuropathy patients after liver transplantation.

Familial amyloidotic polyneuropathy (FAP) has a high prevalence in Portugal, and the most common form of hereditary amyloidosis is caused by an amyloidogenic variant of transthyretin (TTR) with a substitution of methionine for valine at position 30 (V30M). Until now, the available efficient therapy is liver transplantation, when performed in an early phase of the onset of the disease symptoms. However, transplanted FAP patients have a significantly higher incidence of early hepatic artery thrombosis compared with non-FAP transplanted patients. Because FAP was described as an independent risk factor for early hepatic artery thrombosis, more studies to understand the underlying mechanisms involved in this outcome are of the utmost importance. Knowing that the liver is the major site for TTR production, we investigated the biological effects of TTR proteins in the vasculature and on angiogenesis. In this study, we identified genes differentially expressed in endothelial cells exposed to the WT or V30M tetramer. We found that endothelial cells may acquire different molecular identities when exposed to these proteins, and consequently TTR could regulate angiogenesis. Moreover, we show that V30M decreases endothelial survival by inducing apoptosis, and it inhibits migration. These findings provide new knowledge that may have critical implications in the prevention of early hepatic artery thrombosis in FAP patients after liver transplantation.

Familial amyloidotic polyneuropathy (FAP)³ is an inherited autosomal dominant disease that affects primarily the peripheral and autonomic nervous systems. It was first described by Corino de Andrade, in 1952, in the Northern area of Portugal (1), and then many foci of FAP cases were reported worldwide (2–6). FAP is caused by the extracellular deposition of amyloid fibrils composed of transthyretin (TTR) protein. TTR is a homotetrameric plasma protein of approximately 55 kDa produced mainly in the liver and choroid plexus of the brain. The known physiological functions of TTR are the transport of thyroid hormone T4 and retinol, through binding to the retinol-binding protein. To date, approximately 112 mutations in the primary structure of TTR have been discovered of which 98 are amyloidogenic. Among the amyloidogenic variants the substitution of Val for Met at position 30 (V30M) is the most common mutation (7, 8). Several studies suggest that amyloidogenic mutations destabilize the native structure of TTR, thereby inducing conformational changes that lead to dissociation of the tetramers into partially unfolded species which can subsequently self-assemble into amyloid fibrils (9). The proposed mechanism under certain physiological conditions, including temperature, pH, ionic strength, and protein concentration, states that mutant TTR molecules can dissociate into non-native monomers with a distinct compact structure, which in turn can partially unfold forming high molecular mass soluble aggregates (10, 11). Quintas *et al.* have also demonstrated that there is a clear correlation between the stability of the non-native monomer formed upon tetramer dissociation, aggregate formation, and the amyloidogenic potential of different TTR variants (12). Growing evidence has pointed toward a model

* This work was supported by Fundação para a Ciência e Tecnologia Grant PIC/IC/83062/2007.

The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE44856).

¹ Supported by Fundação para a Ciência e Tecnologia (FCT) Fellowship SFRH/BPD/43482/2008.

² To whom correspondence should be addressed: Angiogenesis Unit, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Av. Professor Egas Moniz, 1649-028 Lisbon, Portugal. Tel.: 351-217999411; Fax: 351-217999412; E-mail: sconstantino@fm.ul.pt.

³ The abbreviations used are: FAP, familial amyloidotic polyneuropathy; HAT, hepatic artery thrombosis; HUVEC, human umbilical vein endothelial cell; IPA, Ingenuity Pathway Analysis; OLT, orthotopic liver transplantation; PI, propidium iodide; qRT-PCR, quantitative RT-PCR; TTR, transthyretin.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. Shahin Rafii (Cornell University Medical College, New York). ECs, passage 6–8, were cultured as described (21).

Transthyretin Expression and Purification—Recombinant WT and V30M TTR were produced in an *Escherichia coli* expression system and purified as described previously (22), with minor alterations. WT TTR expression plasmid (pmmHA) was provided by Jeffery Kelly, and V30M TTR expression plasmid (pETM-13) was a courtesy of EMBL-GS (Munich, Germany). *E. coli* BL21+ transformed with the appropriate plasmid was grown overnight in 15-ml starter cultures (LB broth with 50 µg/ml ampicillin, for WT-TTR, or 50 µg/ml kanamycin, for V30M-TTR) at 37 °C and 200 rpm. One-liter cultures (LB broth with antibiotic) were inoculated with 10 ml of starter cultures and incubated at 37 °C and 200 rpm until $A_{600\text{ nm}} = 0.4–0.6$. Protein expression was then induced by adding 0.1 mM isopropyl 1-thio- β -D-galactopyranoside. The purification procedure was applied both for WT and V30M TTR. After 3 h of protein expression cells were centrifuged in a Centrifuge 5810R (Eppendorf) at 9000 rpm for 3 min, at 4 °C, which was followed by resuspension in TBS, pH 7.6, sonication in a Soniprep 150 (MSE), and centrifugation at $14,000 \times g$ for 5 min, at 4 °C. Because TTR is a soluble protein, cell extracts were then precipitated with $(\text{NH}_4)_2\text{SO}_4$ (40% saturation) followed by centrifugation at $12,000 \times g$ for 15 min, at 4 °C. The supernatant was then brought to 90% $(\text{NH}_4)_2\text{SO}_4$ saturation, and precipitated proteins were harvested by centrifugation in the same conditions as the previous one. The pellets were resuspended in ionic exchange buffer A (25 mM Tris-HCl, 1 mM EDTA, pH 8) and dialyzed against the same buffer overnight at 4 °C in dialysis tubing cellulose membrane (average flat width, 76 mm) from Sigma-Aldrich. Dialyzed proteins were separated by ionic exchange chromatography with a Resource Q (GE Healthcare) after equilibration with 95% buffer A and 5% buffer B (25 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 8). TTR variants were collected during a 5–35% buffer B gradient and loaded onto a HiLoad 16/600 Superdex 75 pg (GE Healthcare) equilibrated in PBS. Finally, a tetrameric form of both TTR variants was isolated by gel filtration chromatography as described previously (12), with an analytical Superdex 200 10/300 GL previously equilibrated in Hanks' balanced salt solution.

Transthyretin Quantification and Analysis of the Species in Solution—Protein concentrations were obtained spectrophotometrically at 280 nm using an extinction coefficient of $7.76 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ based on a molecular mass of 55 kDa for TTR ($A_{280\text{ nm}} (1\%) = 14.1 \text{ mg}^{-1} \text{ ml cm}^{-1}$) (11, 12). To assure that just the presence of the WT and V30M TTR tetramer were in the solution, 100 µl of the final purified TTR was injected on an analytical tricorn Superdex 75 (GE Healthcare). Only the samples with just the TTR tetramer were applied to cells.

RNA Isolation, Target Synthesis, and Hybridization to Affymetrix GeneChips—Total RNA was extracted from HUVECs and cultured for 3 h in the presence of the purified WT and V30M TTR proteins at 4 µM, using RNeasy Micro Kit (Qiagen). Three different RNA isolates were used as replicates, and total RNA was

mechanism of amyloidosis cytotoxicity where amyloidogenic mutations in TTR destabilize the native structure into dimers and monomers that rapidly form oligomers and protofibrillar species which are the cytotoxic intermediates (9, 13, 14).

Although several approaches are being developed to induce the clearance of extracellular deposition of TTR (for review, see Ref. 15), so far orthotopic liver transplantation (OLT) has been the treatment of choice for FAP because this organ is the main source of TTR production. Because the liver of FAP patients is usually fully functional, although it produces mutant TTR, in 1995 a new transplant technique, sequential transplantation, or domino liver transplantation, was developed in Portugal, by a team led by Prof. Linhares Furtado (16). In the domino transplantation FAP patients receive an organ, while the organ of the FAP patient is reused for transplantation into another patient with chronic liver disease. A recent prospective monitoring these transplanted FAP patients has shown prolongation of life, and among the different TTR mutations, the most favorable results were obtained for patients with the V30M TTR mutation (17).

Curry Cabral Hospital is presently the most important liver transplantation center in Portugal. Since the first OLT performed in this center in 1992, there have been marked improvements in surgical technique, anesthesia management, immunosuppressive regimen, and medical care of these patients. However, early postoperative thrombotic complications, particularly hepatic artery thrombosis (HAT), remain critical causes of in-hospital morbidity, potentially culminating in acute graft loss (18, 19). A retrospective analysis of 223 OLTs showed that the incidence of early HAT in FAP patients was 7.7-fold higher compared with non-FAP patients. The cause for this higher incidence of HAT could not be justified based on the technical factors analyzed; preoperative details, surgical features, and postoperative variables were similar for patients with or without HAT (20). FAP is therefore an independent risk for early HAT, and it is therefore vital to clarify what are the mechanisms involved in this outcome.

Because the liver is the major site of TTR production, we may hypothesize that the hepatic microenvironment and liver endothelial cells (ECs) respond differentially to the WT or V30M TTR proteins. To date there is no knowledge on the effects of these proteins in EC biology.

In the present study, we used DNA microarray technology to investigate the endothelial global gene expression patterns in response to tetrameric WT and V30M TTR. We report that genes involved in IFN and TNF pathways are significantly modulated by V30M TTR compared with the WT and may have a role in angiogenesis regulation. Accordingly, V30M induces the down-regulation of several pro-angiogenic genes such as *VEGFR1*, *VEGFR2*, *FGF2*, *TGFB2*, and *ANGPT2*. Moreover, our results suggest that V30M decreases EC survival by inducing apoptosis and it inhibits migration. These new insights into the regulation of endothelial function by these proteins may have critical implications in the prevention of early hepatic artery thrombosis in FAP patients after liver transplantation.

extracted using the RNeasy Mini Kit (Qiagen). Concentration and purity were determined by spectrophotometry, and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). RNA was processed for use on Affymetrix GeneChip Human Gene 1.1 ST Array Strips (NuGEN Technologies, San Carlos, CA) Ovation PicoSL WTA System V2, and Encore Biotin Module, according to the manufacturer's protocol. Briefly, 17 ng of total RNA was used in a reverse transcription reaction to obtain first-strand cDNA. After generation and purification of DNA-RNA heteroduplex double-strand cDNA, this was subjected to SPIA amplification. Size distribution of purified SPIA cDNA was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay, followed by fragmentation and end-labeling of 2.5 μ g of SPIA cDNA (Encore Biotin Module, NuGEN) and confirmation using a RNA 6000 Nano Assay. The end-labeled, fragmented cDNA was used in a 130- μ l hybridization mixture containing added hybridization controls (GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips; Affymetrix) and hybridized on array strips for 20 h at 48 °C. Standard post-hybridization wash and double-stain protocols (GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips) were used on an Affymetrix GeneAtlas system, followed by scanning of the array strips. Microarray data were deposited in the Gene Expression Omnibus (GEO) repository at the NCBI under accession number GSE44856.

GeneChip Data Analysis—Scanned arrays were analyzed first with Affymetrix Expression Console software for quality control. Subsequent analysis was carried out with Chipster 2.3.0 (23). Robust multi-array average was used to compute expression values for the six arrays in this study and based on custom cdf file included in hugene11stv1hsentrezg.db as available from Brainarray database (24). Next 33% of all genes with the lowest S.D. from the gene mean were filtered out, leaving 13,224 genes of the original 19,738 genes for further analysis. These were subjected to an empirical Bayes two-group test (25) with Benjamini-Hochberg multiple testing correction and a *p* value cut-off of 0.05, resulting in a list of 436 differentially expressed genes. Ingenuity Pathway Analysis (IPA) (Ingenuity Systems) was used to determine the biological pathways in which the genes that were significantly over- or underexpressed were involved. Our list of significant changed genes, with respective -fold change and *p* values was uploaded within the IPA database and a core analysis was carried out. Canonical pathways analysis identified the pathways from the IPA library that were most significantly associated with the data set. Genes from the data set that met the *p* value cut-off of 5% and were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for the analysis.

Apoptosis Analysis—HUVECs were plated at equal densities, and after 24, 48, 72, 84, or 96 h of incubation with WT and V30M TTR proteins at different concentrations (1, 2, 4, 8, or 10 μ M), cells were stained with Annexin V-FITC (Boehringer Mannheim) and propidium iodide (PI) (Interchim). The percentage of apoptotic cells (Annexin V-positive, PI-negative and -positive) was determined by flow cytometry (FACSCalibur; BD Biosciences) and Flowjo 6.4.7 software. Results are shown as the

percentage of early and late apoptotic cells (Annexin V-positive/PI-negative and Annexin V-positive/PI-positive, respectively).

Wound Healing Assay—HUVECs were plated to confluence and wounds created in the monolayer by scraping the plate with a pipette tip, after the addition of WT and V30M TTR proteins at 4 μ M. Wounded monolayers were photographed immediately after wounding and 7 h later, and the area of the wound was measured. Several regions (*n* = 3) for each treatment done in triplicate were recorded, and the means \pm S.D. of each treatment were calculated after normalizing for wounded area at *t* = 0 and expressed as percentage of wound healing.

Quantitative RT-PCR (qRT-PCR)—Total RNA from HUVECs cultured in the presence of WT and V30M TTR proteins at 4 μ M for 5 or 8 h was isolated using the RNeasy Micro Kit (Qiagen). For each sample, 1 μ g of RNA was reverse transcribed into cDNA (Superscript II Kit; Invitrogen). The mRNA levels of the following targets: *IFITM1* forward, 5'-TCATCCTGTCACCTGGTATTC-GGCTC-3' and reverse, 5'-GTGGGTATAAACTGCTGTATCTAGGG-3'; *BAK1* forward, 5'-GGGTCTATGTTCCCCAGGAT-3' and reverse, 5'-GCAGGGGTAGAGTTGAGCA-3'; *TNF* forward, 5'-GAGCTGTGGGGAGAACAAAAGGA-3' and reverse, 5'-TTGGCCCTTGAAGAGGACCTG-3'; *TNFAIP3(A20)* forward, 5'-CTTGTGGCGCTGAAAACGA-A-3' and reverse, 5'-CCACTGTCCTTCAGGGTCAC-3'; *FOS* forward, 5'-CTCAATGACCCTGAGCCCA-3' and reverse, 5'-TTGCTAATGTTCTTGACCGGC-3'; *VEGFR1* forward, 5'-CCCTCGCCGGAAGTTGTAT-3' and reverse, 5'-GTCAAA-TAGCGAGCAGATTTCTCA-3'; *VEGFR2* forward, 5'-ATTCCTCCCCCGCATCA-3' and reverse, 5'-GCTCGTTGGCGCACTCTT-3'; *FGF2* forward, 5'-AAGAGCGACCCTCACATCAAGCTA-3' and reverse, 5'-TAGCCAGGTAACGGTTAGCACACA-3'; *TGFB2* forward, 5'-GCTTTGGATGCGGCCTATTGCTTT-3' and reverse, 5'-CTCCAGCACAGAAGTTGGCATTGT-3'; *ANGPT2* forward, 5'-AGGACACACCACGAATGGCATCTA-3' and reverse, 5'-TGAATAATTGTCCACCCGCTCCT-3'; *TUB* forward, 5'-CATTGGCAATAGCACAGCCATCCA-3' and reverse, 5'-ACGAGGTCGTTTCATGTGCTCTCA-3'; and *18S* forward, 5'-GCCCTATCAACTTTCGATGGTAGT-3' and reverse, 5'-CCGGAATCGAACCC-TGATT-3' were measured by qRT-PCR performed according to the manufacturer's protocol using Power SYBR® Green (Invitrogen) and an Applied Biosystems 7500 Fast Real-time PCR. The housekeeping gene used to normalize was *18S*. The real-time PCR program consisted of an initial denaturation step at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The relative quantification was performed according to the comparative method ($2^{-\Delta\Delta C_t}$; Applied Biosystems User Bulletin 2P/N 4303859), with WT condition as internal calibrator and *18S* as normalizer. The ΔC_t value for each sample is representative of three different experiments.

RESULTS

Differential Response of ECs to V30M TTR Compared with WT TTR: Human Microarray Study—To characterize the EC response to WT and V30M TTR, we employed DNA microarray analysis, which is a powerful tool for analyzing global changes in gene expression. HUVECs were incubated with WT and V30M TTR tetramers at the final concentration of 4 μ M for

TABLE 1

Top 10 most up-regulated and down-regulated genes induced by V30M TTR compared with WT TTR

A DNA microarray analysis was used to characterize the endothelial cell response to WT and V30M tetrameric TTR proteins. HUVECs were incubated with WT and V30M TTR tetramers at the final concentration of 4 μ M for a period of 3 h. Changes in mRNA expression are given as -fold changes comparing V30M TTR-treated cells versus WT TTR-treated cells. All changes are significant with Benjamini-Hochberg-adjusted *p* values <0.05.

Gene symbol	Gene description	-Fold change
Up-regulated		
<i>SNORD59B</i>	Small nucleolar RNA, C/D box 59B	2.73
<i>POM121L9P</i>	POM121 membrane glycoprotein-like 9, pseudogene	2.35
<i>SNORD49A</i>	Small nucleolar RNA, C/D box 49A	2.18
<i>SNORD113-4</i>	Small nucleolar RNA, C/D box 113-4	2.06
<i>SNORD114-3</i>	Small nucleolar RNA, C/D box 114-3	2.06
<i>SNORD14C</i>	Small nucleolar RNA, C/D box 14C	2.05
<i>HSD17B7</i>	Hydroxysteroid (17 β) dehydrogenase 7	2.02
<i>CLK4</i>	CDC-like kinase 4	2.01
<i>MIR186</i>	MicroRNA 186	2.00
<i>SNORD114-2</i>	Small nucleolar RNA, C/D box 114-2	1.95
Down-regulated		
<i>ANAPC1</i>	Anaphase-promoting complex subunit 1	-2.01
<i>BAK1</i>	BCL2-antagonist/killer 1	-2.04
<i>FAM103A1</i>	Family with sequence similarity 103, member A1	-2.05
<i>OR10G9</i>	Olfactory receptor, family 10, subfamily G, member 9	-2.07
<i>LOC100286979</i>	Similar to anaphase-promoting complex subunit 1	-2.09
<i>EGR1</i>	Early growth response 1	-2.16
<i>HIST1H2BK</i>	Histone cluster 1, H2bk	-2.20
<i>CDC20</i>	Cell division cycle 20 homolog (<i>Saccharomyces cerevisiae</i>)	-2.20
<i>FOSB</i>	FBJ murine osteosarcoma viral oncogene homolog B	-3.67
<i>FOS</i>	FBJ murine osteosarcoma viral oncogene homolog	-3.76

3 h. We chose this concentration because it is the one that resembles the plasma levels of TTR ~25 mg/dl (17, 26). Changes in mRNA expression are given as -fold changes comparing V30M TTR versus WT TTR-treated cells. All changes are significant with Benjamini-Hochberg-adjusted *p* values <0.05. In our study, 213 genes with a ratio of 1.42 or above and 223 genes with a ratio of -1.42 or below were considered up-regulated or down-regulated by V30M, respectively. A list of the top 10 up- or down-regulated genes is presented in Table 1. Of notice are the most down-regulated genes of the data set, *c-fos* and *fosB*, with a -fold change of -3.76 and -3.67, respectively, both belonging to the *fos* gene family. To further investigate the biological relevance of the data obtained from the gene expression microarrays, we used IPA software. The genes considered to have been differentially regulated were uploaded into IPA along with the gene identifiers and corresponding -fold change values. IPA constructs networks that optimize for both interconnectivity and number of focus genes under the constraint of maximal network size. In the graphical representation of a network, genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The intensity of the node color indicates the degree of up (red) or down (green) regulation of a given gene. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed with various labels that describe the nature of the relationship between the nodes. By this analysis, we explored the molecular interactions and biological processes in which the

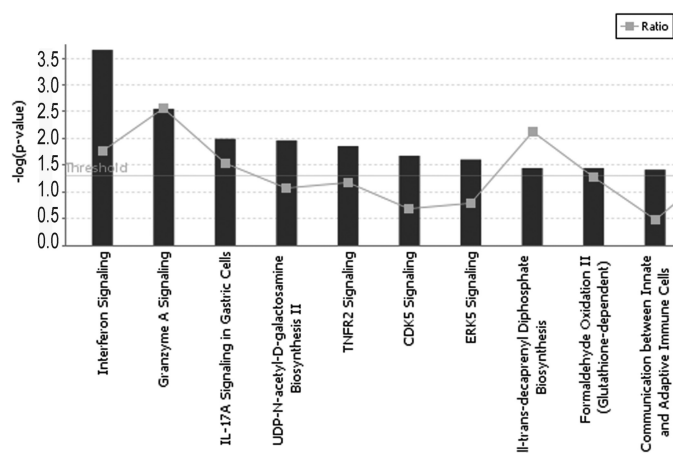


FIGURE 1. IPA software was used to analyze the differentially regulated genes. IPA showing canonical pathways was significantly modulated by V30M TTR compared with WT. Only the 10 pathways with the most significant changes are shown. The *p* value for each pathway is indicated by the bar and is expressed as negative log of Benjamini-Hochberg-adjusted *p* value; threshold is at 1.25 = -log (*p* = 0.05). The gray squares represent the ratio of the number of genes in a given pathway that meet the cut-off criteria divided by the total number of genes that make up that pathway.

genes significantly modified by V30M TTR could be involved. Canonical pathways analysis identified molecular pathways that were most significant to the data set. The top 10 canonical signaling pathways are illustrated in Fig. 1. IFN and TNF are two of the most relevant canonical pathways. In the IFN signaling pathway, the transcript encoding for the T cell protein-tyrosine phosphatase, described as being involved in silencing of VEGFR2 signaling and inhibiting biological responses to VEGF (27), is up-regulated in ECs exposed to V30M TTR when compared with the WT TTR. In contrast, the IFN-induced transmembrane protein 1 (IFITM1), described as regulator of EC sprouting *in vitro* and angiogenesis *in vivo* (28), and the pro-apoptotic Bak1 protein are down-regulated in ECs exposed to V30M TTR. In TNF signaling pathway, the transcripts encoding for the cytokine TNF and for the zing finger protein A20 are up-regulated in V30M exposed ECs. TNF is described as a potent inducer of EC apoptosis (29, 30) whereas A20 has been well established as a dual inhibitor of nuclear factor- κ B (NF- κ B) activation and apoptosis in the TNF receptor 1 signaling pathway. Moreover, in this signaling pathway, the transcript encoding for the proto-oncogene *c-fos* is down-regulated in ECs exposed to V30M. *c-fos* dimerizes with members of Jun family to form AP-1, a transcription factor involved in the regulation of VEGF, one of the most important angiogenic factors (31).

qRT-PCR of Microarray Data—Although microarrays are a powerful tool to study global transcriptional responses, it is important to confirm microarray data. With this objective, HUVECs were cultured in the presence of TTR tetrameric proteins, at 4 μ M, for 5 or 8 h, and total RNA was extracted. Quantitative RT-PCR validated decreased *IFITM1* and *c-fos* expression and increased *A20* and *TNF* expression by the V30M TTR tetramer. We found that *BAK1* expression, one of the top 10 down-regulated genes in the arrays analysis at 3 h, is marginally down-regulated at 5 h but significantly increased at 8 h (Fig. 2)

Transthyretin Proteins Regulate Angiogenesis

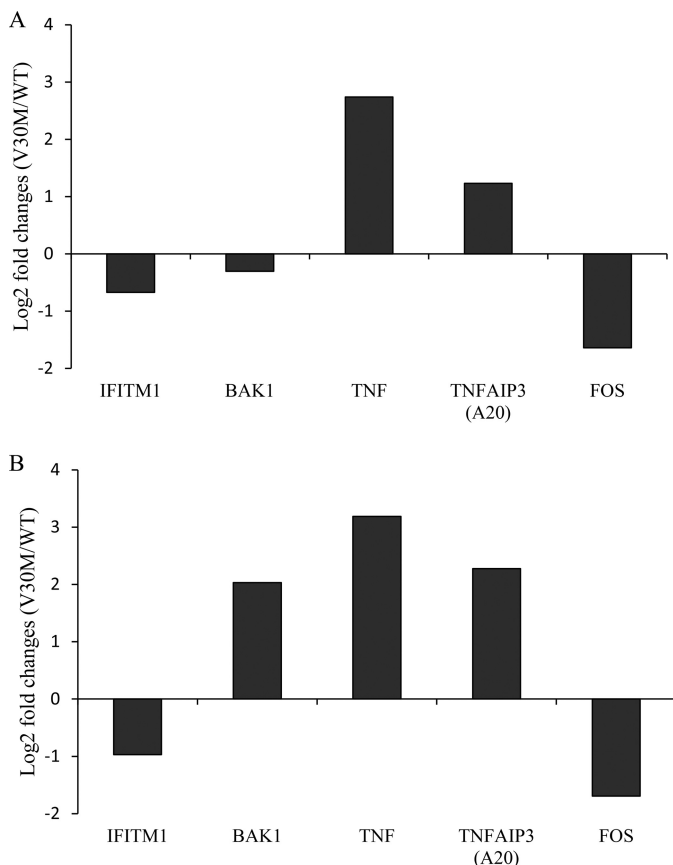


FIGURE 2. Validation of selected microarray targets by qRT-PCR. HUVECs were incubated for 5 h (A) or 8 h (B) with WT and V30M TTR tetrameric proteins. Relative expression of selected targets from the IFN pathway (*IFITM1* and *BAK1*) and from TNFR2 pathway (*TNF*, *TNFAIP3*, *c-fos*) were quantified by qRT-PCR. Data represent the -fold change in gene expression of each target in V30M TTR-treated cells relative to the internal calibrator (WT TTR-treated cells) in triplicate measurements. Chart values are representative of three independent experiments.

in ECs exposed to V30M TTR compared with cells exposed to the WT TTR tetramer.

V30M TTR Tetramers Down-regulate Pro-angiogenic Gene Expression in HUVECs—Because our experimental data suggest that angiogenesis may be modulated by V30M TTR, we investigated the levels of expression of other proteins known to be important in angiogenesis such as VEGFR1, VEGFR2, FGF2, TGFB2, and ANGPT2. According to our microarray data the expression of these targets does not change at 3 h by the V30M TTR tetramer. For that reason, the expression of these selected pro-angiogenic molecules was assessed later. Therefore, HUVECs were cultured in the presence of the WT and V30M TTR tetramer, at 4 μ M, for 5 or 8 h followed by RNA extraction and quantitative RT-PCR analysis. Our results suggest that V30M induces a down-regulation of all selected genes (Fig. 3). Moreover, the expression of *tubulin*, here used as a putative unchanged target by the WT and V30M TTR tetramer, remains identical (Fig. 3A).

V30M TTR Decreases EC Survival by Inducing Apoptosis—According to our findings, the V30M TTR tetramer regulates genes involved in one of the most important pro-angiogenic pathway, VEGF/VEGFR2, described as being fundamental in endothelial survival. For that reason, we assessed the capacity of

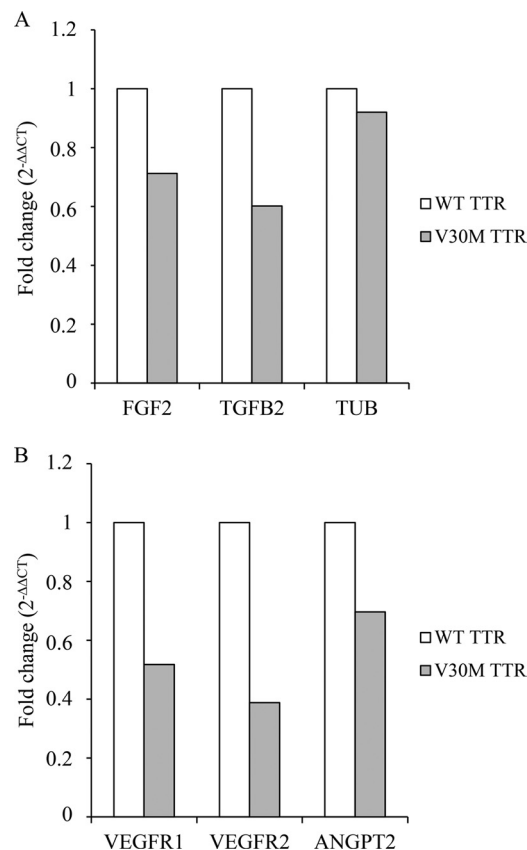


FIGURE 3. V30M TTR tetramers modulate the expression of several pro-angiogenic targets in HUVECs. Cells were incubated for 5 h (A) or 8 h (B) with WT and V30M TTR tetrameric proteins. Relative expression of selected pro-angiogenic genes (*VEGFR1*, *VEGFR2*, *ANGPT2*, *FGF2*, and *TGFB2*) and cytoskeleton-related protein tubulin (*TUB*) was quantified by qRT-PCR. Data represent the -fold change in gene expression of each target in V30M TTR-treated cells relative to the internal calibrator (WT TTR-treated cells) in triplicate measurements. Chart values are representative of three independent experiments.

V30M to induce apoptosis. HUVECs were incubated with WT and V30M TTR tetrameric proteins, at different concentrations, for 24, 48, and 72 h, and then ECs were labeled with Annexin V-FITC and PI. As shown in Fig. 4 A, there is an increase of the percentage of apoptosis in a concentration-dependent manner when HUVECs were cultured with the V30M compared with HUVECs cultured with the WT TTR tetramer. The values obtained for the WT TTR tetramer do not change with the different concentrations or over time (Fig. 4A) and are similar to those observed when HUVECs are cultured in normal medium in the absence of any type of TTR protein (data not shown).

Because 4 μ M is the dose that resembles the plasma levels of TTR \sim 25 mg/dl (17, 26), we investigated whether the percentage of EC apoptosis that was obtained with this dose (Fig. 4A, 4 μ M) could be augmented if the time of incubation increased. HUVECs were incubated with WT or V30M TTR tetrameric proteins at 4 μ M for 24, 48, 72, 84, and 96 h, and as observed (Fig. 4B) V30M TTR tetramer significantly increases the percentage of apoptosis over time in contrast to the WT. Moreover, an additional experimental condition was added (Fig. 4B) where HUVECs were cultured in the presence of both WT and V30M TTR tetrameric proteins (at 2 μ M, each). The same procedure was performed as described above, and according to our results

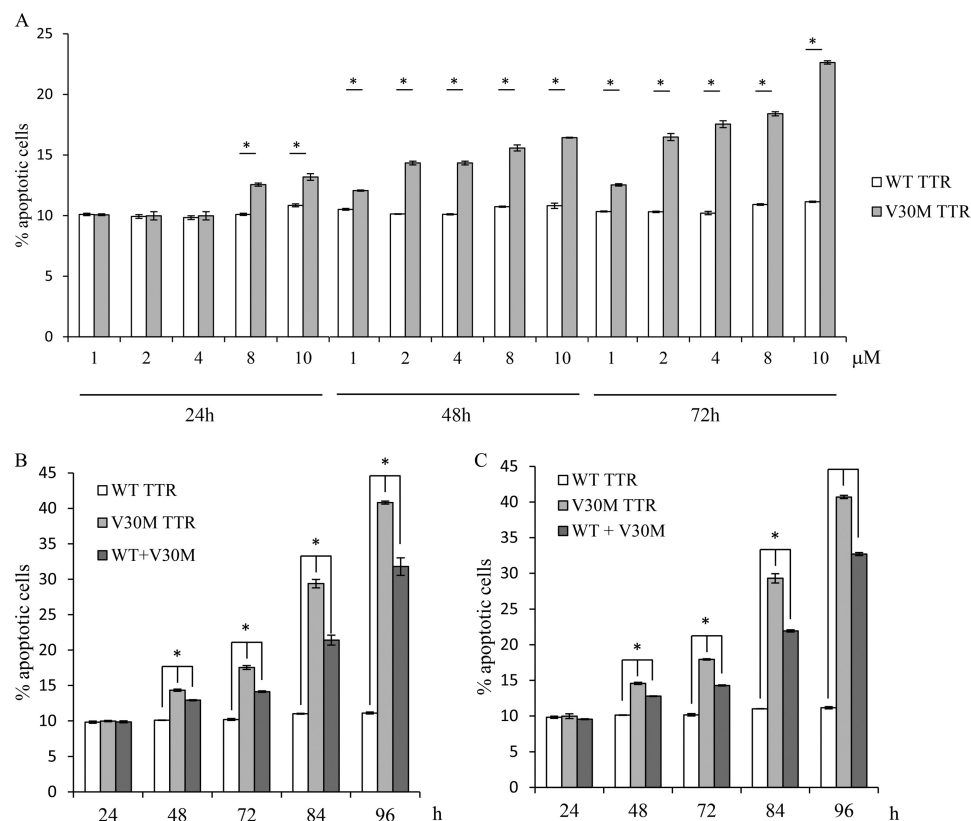


FIGURE 4. V30M TTR decreases endothelial cell survival by inducing apoptosis. A, HUVECs were cultured in the presence of WT and V30M TTR tetrameric proteins at 1, 2, 4, 8, or 10 μ M for 24, 48, and 72 h. After the mentioned incubation periods cells were stained with Annexin V-FITC and PI. B, HUVECs were cultured (i) in the presence of 4 μ M WT TTR; (ii) in the presence of 4 μ M V30M TTR; (iii) in the presence of 2 μ M WT and 2 μ M V30M TTR for 24, 48, 72, 84, and 96 h. After the mentioned incubation periods cells were stained with Annexin V-FITC and PI. C, HUVECs were cultured (i) in the presence of 4 μ M WT TTR; (ii) in the presence of 4 μ M V30M TTR; (iii) in the presence of 2 μ M WT and 2 μ M V30M TTR for 24, 48, 72, 84, and 96 h. Every 24 h, the medium was removed, and fresh medium was added with the respective tetramers. After the incubation periods cells were stained with Annexin V-FITC and PI. A–C, values are given as the percentage of apoptotic cells (Annexin V-positive, PI-negative and -positive) in culture. Data are shown as mean \pm S.D. of triplicate cultures and are representative of three independent experiments. A, *, $p < 0.0005$; B and C, *, $p < 0.00025$.

the percentage of apoptosis after 48 h is significantly superior when ECs are cultured simultaneously in the presence of both types of proteins (WT and V30M) compared with the values obtained when ECs are cultured exclusively in the presence of WT, but significantly inferior compared with those obtained when ECs are cultured exclusively in the presence of V30M. Furthermore, we investigated whether the percentage of EC apoptosis that was obtained in this particular condition could be changed by the addition of fresh tetramer every 24 h over 96 h. Accordingly, HUVECs were incubated with WT or V30M TTR tetrameric proteins at 4 μ M or both proteins (at 2 μ M) for 24, 48, 72, 84, and 96 h. Every 24 h, the medium was removed, and fresh medium was added with the respective tetramers. ECs were labeled with Annexin V-FITC and PI and the percentage of apoptosis analyzed at indicated times (Fig. 4C). As observed in Fig. 4C, the results were similar to those obtained when tetramers were added uniquely at the beginning of the experiment (Fig. 4B).

V30M TTR Tetramer Inhibits EC Migration—We also investigated the effect of the V30M TTR tetramer in EC migration because our results suggest that TTR may regulate angiogenesis, and we know that in this process cell migration is essential. Confluent HUVEC monolayers were exposed to either protein, at 4 μ M, and an *in vitro* wound healing assay was performed. The EC migratory response to wounding was measured. As

represented in Fig. 5, ECs in the presence of the WT achieved almost 85% wound closure after 7 h, whereas V30M TTR inhibited closure by approximately 1.4-fold (60% wound closure). At 7 h after wounding, neither type of TTR protein induces apoptosis (data not shown), suggesting that the result obtained is independent of a putative effect of V30M in EC survival.

DISCUSSION

Orthotopic liver transplant for FAP has become the usual treatment of choice for this fatal disease. However, transplanted FAP patients have a significantly higher incidence of early hepatic artery thrombosis compared with non-FAP transplanted patients. Because FAP was described as an independent risk factor for early HAT, more studies aiming to clarify this phenomenon are needed to develop specific prevention strategies for this population. Knowing that the livers of FAP patients are anatomically and functionally normal except for the production of the mutant TTR, we hypothesized that ECs may acquire different molecular identities when exposed to WT or mutant TTR proteins, and consequently TTR could regulate angiogenesis. To the best of our knowledge, the biological effects of TTR proteins in the vasculature and on angiogenesis were unknown.

To address this issue, we investigated global gene expression of HUVECs cultured in the presence of the WT and V30M

Transthyretin Proteins Regulate Angiogenesis

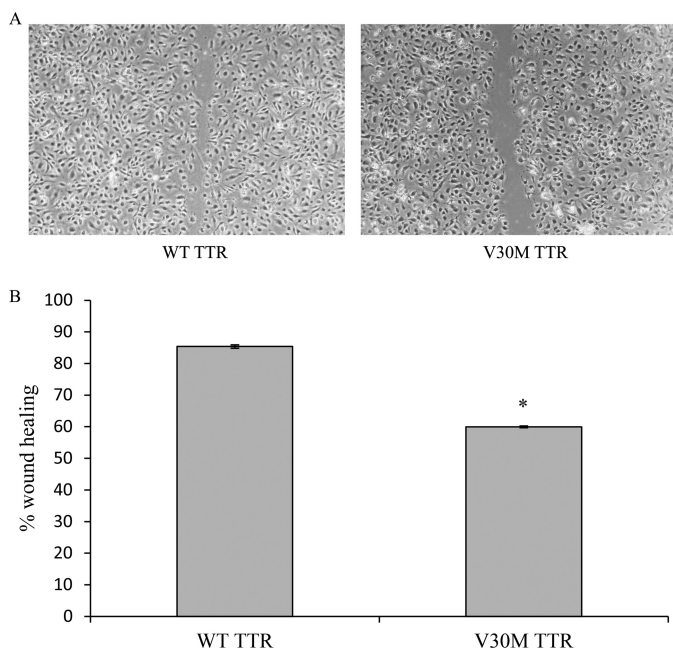


FIGURE 5. V30M TTR tetramer inhibits endothelial cell migration. *A*, HUVECs were cultured until confluent, and the monolayer was scraped to initiate wounding immediately after the addition of TTR tetrameric proteins at 4 μ M. Monolayers were followed over time and the wound area was measured at $t = 0$ h and $t = 7$ h. Magnification, $\times 10$. *B*, quantitation of results from several regions ($n = 3$) for each treatment done in triplicate was recorded, and the means \pm S.D. of each treatment were calculated after normalizing for wounded area at $t = 0$ and expressed as percentage of wound healing. *, $p < 0.0002$.

tetrameric TTR proteins, at 4 μ M, a concentration similar to the one found physiologically. The first key finding of this work is the identification of differentially expressed genes in ECs exposed to the WT or V30M TTR tetramer. By microarray analysis, we found that the expression of a total of 436 genes differed significantly between the ECs exposed to the WT and V30M tetramer. To identify mechanisms behind experimental observations of changes in gene expression, our data sets were analyzed through the use of IPA canonical pathways. The IFN and TNF signaling pathways were identified as canonical pathways that were most significant to the data set. Interestingly, all of the mediators observed to be up- or down-regulated in these particular pathways, namely *T cell protein-tyrosine phosphatase*, *IFITM1*, *BAK1*, *TNF*, *A20*, and *c-fos*, are involved in endothelial cell survival and angiogenesis regulation, the second key finding of this work. To validate our microarray data set, qRT-PCR was performed. Consistently, the V30M TTR tetramer induces a down-regulation of the transcript levels of *IFITM1* and *c-fos* and an up-regulation of *TNF* and *A20*, in HUVECs, at 5 or 8 h of exposure. *IFITM1* and *c-fos* are both involved in angiogenesis regulation. *IFITM1* was found to be rapidly induced as EC sprout *in vitro* and form luminized vessels. Using a vascular bed xenotransplant model, a role for the human *IFITM1* was identified during *in vivo* angiogenesis (28). *IFITM1* was described as a molecule that may regulate the transition of ECs from a quiescent to an angiogenic state (28). Because we found that V30M tetrameric protein induces a decrease in *IFITM1* expression, this could be related to an anti-angiogenic response that could be in agreement with our results that sug-

gest that V30M inhibits EC migration. The protein *c-fos* heterodimerizes with Jun proteins to form the transcription factor AP-1. According to our microarray analysis, *Jun* expression is not significantly changed by V30M, a finding that was confirmed by qRT-PCR (data not shown). Taken together, our results suggest that the expression of AP-1 may be consequently decreased in HUVECs exposed to V30M. One of the genes regulated by AP-1 is one of the most important angiogenic factors, *VEGF* (31). Moreover, it was also described that, in ECs, AP-1 is also involved in the transcription of interleukin 8 (IL-8), which acts in an autocrine fashion to suppress apoptosis and facilitate cell proliferation and migration (32). In agreement with our results that suggest that V30M induces *AP-1* down-regulation, our observations shown that V30M induces apoptosis and inhibits EC migration, the third key finding of our work.

The proteins TNF and A20 are mainly involved in the regulation of EC survival. *TNF*, up-regulated by V30M TTR tetramer, is a potent inducer of EC apoptosis, consistent with the effect of V30M in inducing EC apoptosis. Concerning *A20*, it is difficult to predict its biological effect in ECs exposed to V30M, where we found that its expression levels are up-regulated, because this protein has a dual role. Although *A20* inhibits apoptosis mediated by the TNF signaling, it paradoxically inhibits NF- κ B. In most cell types, it has been well established that activation of NF- κ B antagonizes apoptotic cell death triggered by TNF signaling. Moreover, our results suggest that *BAK1*, one of the 10 top down-regulated genes by V30M TTR in our array analysis (performed at 3 h), is marginally down-regulated at 5 h. This suggests that even though the levels of *BAK1* significantly decrease at 3 h of V30M TTR exposure, this slight effect is seen later, at 5 h, and interestingly, at 8 h, the levels of expression of *BAK1* transcripts are significantly increased by V30M TTR compared with the WT tetramer. *BAK1* is a pro-apoptotic gene, and its up-regulation by V30M TTR is consistent with our results showing that V30M induces HUVECs to undergo apoptosis. According to our findings for *BAK1*, we hypothesized that other molecular targets playing a role in angiogenesis and that do not appear as significantly altered in our microarray data set (performed at 3 h) could be differentially expressed by V30M TTR tetramer later on. Therefore, we selected several genes such as *VEGFR1*, *VEGFR2*, *FGF2*, *TGFB2*, and *ANGPT2*. The receptors 1 and 2 of VEGF (*VEGFR1* and *VEGFR2*) are the main mediators of VEGF-induced endothelial proliferation, survival, migration, tubular morphogenesis, and sprouting in adults (33, 34). Moreover, it has been long recognized that *FGF2*, *TGFB2*, and *ANGP2* are important in angiogenesis inducing migration and proliferation of ECs, stimulating tube formation and sprouting of new blood vessels (35). Here, we found that HUVECs present a down-regulation of the expression of all of these angiogenic players after 5 or 8 h of exposure to V30M compared with the WT TTR tetramer. Therefore, our results consistently raise the possibility that TTR proteins differentially regulate endothelial gene expression, conferring different molecular identities to ECs. Accordingly, V30M TTR tetramer decreases the expression levels of several genes involved in endothelial proliferation, survival, and migration and could negatively modulate angiogen-

esis compared with the WT TTR tetramer. Our results provide the important insight that TTR protein plays a key regulatory role in angiogenesis whereby the mutant V30M or WT tetramer differentially activates ECs, inducing a specific pattern of gene expression. According to the cellular pathways and molecular targets that are expressed, distinct vascular responses will be dictated by the V30M or the WT TTR tetramer.

To the best of our knowledge, the evidence that FAP patients could develop diseases associated with angiogenesis deficiency has not been previously noted. We can hypothesize that the normal life span of the FAP patient minimizes the chance of the development of angiogenesis-related diseases. However, FAP was recently identified as an independent risk factor for early thrombotic complications and specifically for HAT following OLT often culminating in acute graft loss (20). The cause of this phenomenon is not known. In light of presented data, we can hypothesize that the sudden change in serum levels of TTR V30M to WT can be challenging to FAP donor ECs. Knowing that FAP is an autosomal dominant disease, it is expectable that in the majority of FAP patients, their ECs could be exposed simultaneously to the WT and V30M proteins, but even so, our results suggest that ECs are biologically affected by V30M because an induction of apoptosis is observed in the presence of both tetrameric proteins. However, according to our results suggesting that the percentage of apoptosis is significantly different among ECs exposed to both types of TTR tetrameric proteins and ECs exposed exclusively to V30M tetrameric protein, we expect that ECs from homozygotic or heterozygotic FAP patients for V30M may respond differentially. Nevertheless, homozygotic or heterozygotic FAP patients for V30M were exposed to TTR V30M through life, which induced a low angiogenic potential. Few hours after OLT, the microenvironment is altered, and the vasculature should respond to a WT protein (produced by the new transplanted liver), which induces different biological pathways and gene expression favoring angiogenesis and EC proliferation. Also, as reported by our surgeons, HAT occurs mainly in the donor side of vascular anastomosis (data not shown). This fact is in accordance with our description. However, HAT is not a concern of FAP patients only, occurring also in non-FAP receptors. This means that local factors must be taken into account. Nonetheless, an existing vascular anastomosis is a huge stress factor for angiogenesis. It may partially explain why the thrombosis events occur in the anastomosis area, not at distant sites. This study highlighted the severe problem of high incidence HAT in FAP recipients during OLT. We consider that with this work new knowledge has been introduced in this area, but the problem remains. Further investigation is needed to clarify this matter, mainly which interventional treatments are required.

Acknowledgment—We thank Mike Saure, from The Scripps Research Institute, the technical support with the procedure of production and purification of transthyretin proteins.

REFERENCES

1. Andrade, C. (1952) A peculiar form of peripheral neuropathy: familial atypical generalized amyloidosis with special involvement of the peripheral

2. Benson, M. D., Wallace, M. R., Tejada, E., Baumann, H., and Page, B. (1987) Hereditary amyloidosis: description of a new American kindred with late onset cardiomyopathy. *Appalachian amyloid. Arthritis Rheum.* **30**, 195–200
3. Dwulet, F. E., and Benson, M. D. (1984) Primary structure of an amyloid prealbumin and its plasma precursor in a hereditary familial polyneuropathy of Swedish origin. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 694–698
4. Nakazato, M., Kangawa, K., Minamino, N., Tawara, S., Matsuo, H., and Araki, S. (1984) Identification of a prealbumin variant in the serum of a Japanese patient with familial amyloidotic polyneuropathy. *Biochem. Biophys. Res. Commun.* **122**, 712–718
5. Palacios, S. A., Bittencourt, P. L., Cançado, E. L., Farias, A. Q., Massarollo, P. C., Mies, S., Kalil, J., and Goldberg, A. C. (1999) Familial amyloidotic polyneuropathy type 1 in Brazil is associated with the transthyretin V30M variant. *Amyloid* **6**, 289–291
6. Suhr, O. B., Svendsen, I. H., Andersson, R., Danielsson, A., Holmgren, G., and Ranløv, P. J. (2003) Hereditary transthyretin amyloidosis from a Scandinavian perspective. *J. Intern. Med.* **254**, 225–235
7. Saraiva, M. J., Birken, S., Costa, P. P., and Goodman, D. S. (1984) Amyloid fibril protein in familial amyloidotic polyneuropathy, Portuguese type: definition of molecular abnormality in transthyretin (prealbumin). *J. Clin. Invest.* **74**, 104–119
8. Benson, M. D., and Kincaid, J. C. (2007) The molecular biology and clinical features of amyloid neuropathy. *Muscle Nerve* **36**, 411–423
9. Hou, X., Aguilar, M. I., and Small, D. H. (2007) Transthyretin and familial amyloidotic polyneuropathy. *FEBS J.* **274**, 1637–1650
10. Quintas, A., Saraiva, M. J., and Brito, R. M. (1997) The amyloidogenic potential of transthyretin variants correlates with their tendency to aggregate in solution. *FEBS Lett.* **418**, 297–300
11. Quintas, A., Saraiva, M. J., and Brito, R. M. (1999) The tetrameric protein transthyretin dissociates to a non-native monomer in solution: a novel model for amyloidogenesis. *J. Biol. Chem.* **274**, 32943–32949
12. Quintas, A., Vaz, D. C., Cardoso, I., Saraiva, M. J., and Brito, R. M. (2001) Tetramer dissociation and monomer partial unfolding precedes protofibril formation in amyloidogenic transthyretin variants. *J. Biol. Chem.* **276**, 27207–27213
13. Sousa, M. M., Cardoso, I., Fernandes, R., Guimarães, A., and Saraiva, M. J. (2001) Deposition of transthyretin in early stages of familial amyloidotic polyneuropathy: evidence for toxicity of nonfibrillar aggregates. *Am. J. Pathol.* **159**, 1993–2000
14. Reixach, N., Deechongkit, S., Jiang, X., Kelly, J. W., and Buxbaum, J. N. (2004) Tissue damage in the amyloidoses: transthyretin monomers and nonnative oligomers are the major cytotoxic species in tissue culture. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 2817–2822
15. Almeida, M. R., and Saraiva, M. J. (2012) Clearance of extracellular misfolded proteins in systemic amyloidosis: experience with transthyretin. *FEBS Lett.* **586**, 2891–2896
16. Furtado, A., Tomé, L., Oliveira, F. J., Furtado, E., Viana, J., and Perdigoto, R. (1997) Sequential liver transplantation. *Transplant. Proc.* **29**, 467–468
17. Benson, M. D. (2013) Liver transplantation and transthyretin amyloidosis. *Muscle Nerve* **47**, 157–162
18. Stange, B. J., Glanemann, M., Nuessler, N. C., Settmacher, U., Steinmüller, T., and Neuhaus, P. (2003) Hepatic artery thrombosis after adult liver transplantation. *Liver Transpl.* **9**, 612–620
19. Mueller, A. R., Platz, K. P., and Kremer, B. (2004) Early postoperative complications following liver transplantation. *Best Pract. Res. Clin. Gastroenterol.* **18**, 881–900
20. Bispo, M., Marcelino, P., Freire, A., Martins, A., Mourão, L., and Barroso, E. (2009) High incidence of thrombotic complications early after liver transplantation for familial amyloidotic polyneuropathy. *Transpl. Int.* **22**, 165–171
21. Sofia Vala, I., Martins, L. R., Imaizumi, N., Nunes, R. J., Rino, J., Kuonen, F., Carvalho, L. M., Rüegg, C., Grillo, I. M., Barata, J. T., Mareel, M., and Santos, S. C. (2010) Low doses of ionizing radiation promote tumor growth and metastasis by enhancing angiogenesis. *PLoS ONE* **5**, e11222
22. Foss, T. R., Kelker, M. S., Wiseman, R. L., Wilson, I. A., and Kelly, J. W. (2005) Kinetic stabilization of the native state by protein engineering: eral nerves. *Brain* **75**, 408–427

- implications for inhibition of transthyretin amyloidogenesis. *J. Mol. Biol.* **347**, 841–854
23. Kallio, M. A., Tuimala, J., Hupponen, T., Klemelä, P., Gentile, M., Scheinin, I., Koski, M., Kåki, J., and Korpelainen, E. (2011) Chipster: user-friendly analysis software for microarray and other high-throughput data. *BMC Genomics* **12**, 507
24. Sandberg, R., and Larsson, O. (2007) Improved precision and accuracy for microarrays using updated probe set definitions. *BMC Bioinformatics* **8**, 48
25. Smyth, G. K. (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **3**, article 3
26. Buxbaum, J. N., and Reixach, N. (2009) Transthyretin: the servant of many masters. *Cell Mol. Life Sci.* **66**, 3095–3101
27. Ivaska, J., and Mattila, E. (May 13, 2008) Method for inhibiting or stimulating angiogenesis in an individual. U. S. Patent 20,100,160,228
28. Popson, S. A., and Hughes, C. C. (2010) A role for IFITM proteins in angiogenesis. *FASEB J.* **24**, 750–751
29. Robaye, B., Mosselmans, R., Fiers, W., Dumont, J. E., and Galand, P. (1991) Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells *in vitro*. *Am. J. Pathol.* **138**, 447–453
30. Polunovsky, V. A., Wendt, C. H., Ingbar, D. H., Peterson, M. S., and Bitterman, P. B. (1994) Induction of endothelial cell apoptosis by TNF α : modulation by inhibitors of protein synthesis. *Exp. Cell Res.* **214**, 584–594
31. Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C., and Abraham, J. A. (1991) The human gene for vascular endothelial growth factor: multiple protein forms are encoded through alternative exon splicing. *J. Biol. Chem.* **266**, 11947–11954
32. Abdel-Malak, N. A., Srikant, C. B., Kristof, A. S., Magder, S. A., Di Battista, J. A., and Hussain, S. N. (2008) Angiopoietin-1 promotes endothelial cell proliferation and migration through AP-1-dependent autocrine production of interleukin-8. *Blood* **111**, 4145–4154
33. Orecchia, A., Lacal, P. M., Schietroma, C., Morea, V., Zambruno, G., and Failla, C. M. (2003) Vascular endothelial growth factor receptor-1 is deposited in the extracellular matrix by endothelial cells and is a ligand for the $\alpha 5 \beta 1$ integrin. *J. Cell Sci.* **116**, 3479–3489
34. Ferrara, N., Gerber, H. P., and LeCouter, J. (2003) The biology of VEGF and its receptors. *Nat. Med.* **9**, 669–676
35. Distler, J. H., Hirth, A., Kurowska-Stolarska, M., Gay, R. E., Gay, S., and Distler, O. (2003) Angiogenic and angiostatic factors in the molecular control of angiogenesis. *Q. J. Nucl. Med.* **47**, 149–161

Transthyretin Proteins Regulate Angiogenesis by Conferring Different Molecular Identities to Endothelial Cells

Raquel J. Nunes, Paula de Oliveira, Ana Lages, Jörg D. Becker, Paulo Marcelino, Eduardo Barroso, Rui Perdigoto, Jeffery W. Kelly, Alexandre Quintas and Susana Constantino Rosa Santos

J. Biol. Chem. 2013, 288:31752-31760.

doi: 10.1074/jbc.M113.469858 originally published online September 12, 2013

Access the most updated version of this article at doi: [10.1074/jbc.M113.469858](https://doi.org/10.1074/jbc.M113.469858)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 33 references, 9 of which can be accessed free at <http://www.jbc.org/content/288/44/31752.full.html#ref-list-1>