Proteome Analysis of Cultivated Vascular Smooth Muscle Cells from a CADASIL Patient

Saara Ihalainen,[1,2] Rabah Soliymani,[1] Erika Iivanainen,[4,5] Kati Mykkänen,[6] Annele Sainio,[4] Minna Pöyhönen,[3] Klaus Elenius,[4,7,10] Hannu Järveläinen,[4,11] Matti Viitanen,[8,12] Hannu Kalimo,[2,9,13] and Marc Baumann[1]

[1]Protein Chemistry Unit, Institute of Biomedicine/Anatomy, [2]Department of Pathology, and [3]Department of Medical Genetics Haartman Institute, University of Helsinki, Helsinki, Finland; [4]Department of Medical Biochemistry and [5]Molecular Biology, Turku Graduate School of Biomedical Sciences; [6]Department of Medical Genetics, [7]Medicity Research Laboratories, [8]Department of Geriatrics, and [9]Department of Pathology, University of Turku, Turku, Finland; [10]Department of Oncology and Radiotherapy and [11]Department of Medicine, Turku University Central Hospital, Turku, Finland; [12]Division of Clinical Geriatrics, Karolinska Institutet, Stockholm, Sweden;[13]Department of Genetics and Pathology, University of Uppsala, Uppsala, Sweden

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a vascular dementing disease caused by mutations in the *NOTCH3* gene, most which are missense mutations leading to an uneven number of cysteine residues in epidermal growth factor–like repeats in the extracellular domain of Notch3 receptor (N3ECD). CADASIL is characterized by degeneration of vascular smooth muscle cells (VSMC) and accumulation of N3ECD on the VSMCs of small and middle-sized arteries. Recent studies have demonstrated that impairment of Notch3 signaling is not the primary cause of the disease. In the present study we used proteomic analysis to characterize the protein expression pattern of a unique material of genetically genuine cultured human CADASIL VSMCs. We identified 11 differentially expressed proteins, which are involved in protein degradation and folding, contraction of VSMCs, and cellular stress. Our findings indicate that misfolding of Notch3 may cause endoplasmic reticulum stress and activation of unfolded protein response, leading to increased reactive oxygen species and inhibition of cell proliferation. In addition, upregulation of contractile proteins suggests an alteration in the signaling system of VSMC contraction. The accumulation of N3ECD on the cell surface possibly upregulates the angiotensin II regulatory feedback loop and thereby enhances the readiness of the cells to respond to angiotensin II stimulation.

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INTRODUCTION

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary arteriopathy causing recurrent ischemic strokes and finally vascular dementia. The disease is characterized by vascular smooth muscle cell (VSMC) degeneration caused by mutations in the extracellular domain of the Notch3 receptor molecule (N3ECD) resulting in accumulation of N3ECD as well as granular osmiophilic material (GOM) on the VSMCs of small and middle-sized arteries (1). Consequently the walls of the affected arteries in the cerebral white matter become markedly thickened, mainly because of accumulation of type I collagen, resulting in stenosis with subsequent white matter lesions and lacunar infarcts.

More than 140 different mutations have been identified in the *NOTCH3* gene that encodes Notch3 protein. An overwhelming majority of these are missense point mutations that result in an odd number of cysteine residues in the affected epidermal growth factor (EGF)-like repeats in N3ECD (2). These mutations lead to a disruption of the native Notch3 structure and cause misfolding of the protein (3).

Notch3 is an evolutionarily conserved single transmembrane-spanning protein with a large extracellular domain. During development Notch3 regulates cell differentiation and is expressed in various tissues. In human adults the role of Notch3 is unknown and its expression has been demonstrated only in VSMCs (1).

Upon binding of its ligands, members of Jagged and Delta families, Notch3 undergoes two proteolytic cleavages. First Notch3 is cleaved on the extracellular side by the TNFα-converting enzyme (4,5) followed by cleavage within the plasma membrane by γ-secretase, which releases the intracellular domain (N3ICD) of the receptor (6). After the cleavage N3ICD translocates to the cell nucleus and interacts with the DNA-binding protein RBP-Jκ. This complex

Address correspondence and reprint requests to *Saara Ihalainen, Protein Chemistry Unit, Biomedicum Helsinki, P.O.box 63, 00014 University of Helsinki, Finland. Phone: +358 9 191 25203; Fax: +358 9 191 25206; E-mail: saara.ihalainen@helsinki.fi*
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regulates the transcription of target genes, mainly the hairy/enhancer of split (HES-1) genes (7).

Recent studies on wild-type and mutated Notch3 transfected cells in vitro showed that the classic Notch3 signaling, i.e., the activation of transcription factor RBJ-Jκ and HES-1 genes, was impaired only when the mutations were located in the ligand-binding site on EGF repeats 10-11 in N3ECD (8–10), whereas the tested pathogenic mutations outside the binding site did not affect the signaling. This is remarkable, because virtually all of the more than 140 different mutations, irrespective of their site, cause a similar clinical phenotype. Therefore it can be assumed that direct impairment of Notch3 signaling is not solely the cause of the disease.

In the present work we used proteomic analysis of protein expression differences in cultured VSMCs isolated from the umbilical cords of one newborn with CADASIL and five healthy controls. These human cell lines are unique, genetically genuine VSMCs expressing only the endogenous mutated or normal Notch3 receptor. We detected differences in the expression level of proteins involved in protein degradation and folding, interaction with cytoskeletal proteins and contraction of VSMCs, and cellular stress. These results suggest that the misfolding of Notch3 causes ER stress and activation of unfolded protein response (UPR) leading to increased formation of reactive oxygen species (ROS) and inhibition of cell proliferation. In addition, upregulation of VSMC contractile proteins suggests that the accumulation of Notch3 on the cell surface may upregulate the angiotensin II regulatory feedback loop and thereby enhance the ability of the cells to respond to angiotensin II stimulation.

MATERIALS AND METHODS

Cell Culture

The VSMC line was established from the umbilical cords of three newborn babies whose mothers had CADASIL with a molecular genetically verified *NOTCH3* C475T (R133C) mutation, which is the predominant mutation in Finnish CADASIL patients (11), and three newborn babies of healthy mothers. The acquisition of the umbilical cords was approved by the Ethical Board of the Hospital District of Varsinais-Suomi and Turku University Hospital, and the National Authority for Medicolegal Affairs.

The cells were isolated from the umbilical cord by collagenase digestion as described previously (12,13) or by surgical preparation of blood vessel walls. Strips (2–3 mm long) of blood vessel walls were transferred onto 30-mm petri dishes and were allowed to dry ~20 min before addition of culture medium. The cells were cultured in DMEM with 10% fetal bovine serum in the presence of L-glutamine and penicillin/streptomycin (both from GIBCO Invitrogen Corporation, Paisley, UK) in 5% CO_2 at 37°C. To partially immortalize the cell lines the cells were infected with a human papilloma virus construct p6/p7 in early passage (p3-p5). The infection was verified by culturing the cells in the presence of G418 (400 µg/mL) for a 10-day period. All the cell cultures were screened negative for mycoplasma by VenorGeM mycoplasma detection kit (Minerva Biolabs GmbH, Berlin, Germany). The cells used in the expression studies were at passage 7.

Characterization of the Cells

Immunocytochemistry. For characterization of the cell type, the cells were fixed in –20°C methanol (HPLC-grade, Rathburn Chemicals Ltd, Walkerburn, Scotland), blocked with 3% BSA (Sigma, St Louis, MO, USA) in tris-buffered saline, 0.1% Tween-20 (Sigma-Aldrich Chemie GmbH, Steinheim, UK), and then incubated with mouse monoclonal anti–smooth muscle β-actin antibody (Sigma) (dilution 1:40 000) followed by FITC-conjugated secondary anti-mouse IgG antibody (Molecular Probes Inc, Leiden, the Netherlands) (dilution 1:250).

Genotyping. The genotype (presence or absence of the C475T mutation) was verified by amplifying exon 4 from *NOTCH3* gene by PCR and digesting the PCR product with a restriction enzyme MspA1I. Mutation V475 deletes one MspA1I restriction site producing larger restriction fragment as compared to wild-type Notch3.

Western blot. To determine if these cells express Notch3 receptor, 60 µg of the total protein lysates were separated by 6% SDS-PAGE in reducing conditions and transferred from the gels to PVDF-membranes (Millipore Corporation, Bedford, MA, USA) in a semidry blotter for 1.5 h at 45 mA in 25 mM Tris, 192 mM glycine, pH 8.3 (Bio-Rad Laboratories, Hercules, CA, USA) with 20% methanol. The membranes were blocked with 5% (w/v) nonfat dry milk. The N3ECD and full-length Notch3 were detected with a mouse monoclonal antibody clone 1E4, an antibody raised against N3ECD, (a kind gift from Dr. A. Joutel, dilution 1:1000) overnight at 4°C, and with horse radish peroxidase–linked secondary antibody goat anti-mouse (dilution 1:100 000) (Bio-Rad Laboratories). Immunoreactive bands were visualized with ECL plus western blotting kit (Amersham Pharmacia Biotech UK Ltd, Little Chalfent, UK) and scanned with Typhoon 9400 fluorescence scanner (Amersham Biosciences, Sunnyvale, CA, USA).

Two-Dimensional Gel Electrophoresis

The cells were lysed on ice in lysis buffer containing 7 M urea, 2 M thiourea, 30 mM tris, and 4% CHAPS. The cell suspensions were sonicated 3 × 45 s and centrifuged at 13 000 g for 10 min at 4°C. The resulted cell debris was discarded and the total protein amount in the supernatant was measured by 2-D Quant kit (Amersham Biosciences Co, San Francisco, CA, USA). We mixed 50 µg (for analytical gels) or 100–150 µg (preparative gels) of protein suspension with rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% IPG-buffer (pH 3-10 or pH 4-7, Amersham Biosciences AB, Uppsala, Sweden), and 1% DTT. The IEF was performed on an IPGphor (Amersham Pharmacia Biotech UK Ltd) with

18 cm IPG-strips, pH-range 3-10 or 4-7 (Amersham Biosciences AB). The strips were rehydrated passively with the sample for at least 12 h and the actual focusing performed according to volt h (34 000 Vhr). After IEF the strips were incubated for 15 min in equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 2.0% SDS, 30% glycerol in the presence of 1% DTT). The second equilibration was performed in the same buffer with 4% iodoacetamide. The equilibrated strips were placed on top of the 12% acrylamide gels containing SDS. Electrophoresis was performed with Powerpac 3000 (Bio-Rad Laboratories) at the beginning with 100 V and after the sample had entered the gel, proceeded with 200 V until the front line reached the end of the gel. The gels were silver stained with the Silver stain plus kit (Amersham Biosciences AB) according to the protocol for mass spectrometry in a Hoefer Processor Plus gel stainer (Amersham Pharmacia Biotech UK Ltd).

Image Analysis and Data Processing

The images of protein spots were scanned (Scanner GS800, Bio-Rad Laboratories), and spot intensities obtained using the image analysis software PDQuest (Bio-Rad, Hercules, CA, USA). The images were normalized according to the total density of the gel, and spot intensities were compared between CADASIL and control groups, which were composed of three replicates of each sample. Statistical significance of intensity differences was analyzed using the Student *t* test (PDQuest) and One-Way ANOVA (SPSS).

In-Gel Digestion and Protein Identification

Protein spots were manually excised and cut into small pieces. The spots were destained, reduced, and alkylated. Into each spot was added 0.05 µg of trypsin (sequencing grade; Promega, Madison, WI, USA) in 0.1 M NH_4HCO_3 and incubated overnight at 37°C. Tryptic peptides were extracted from the gel pieces with 5% formic acid. The peptides from the eluates were bound to RP C-18 columns (ZipTip, Millipore, Bedford, MA, USA) and eluted with 1–2 µL of MALDI-matrix (33% ACN – 0.1% TFA saturated with CHCA). The samples were analyzed by MALDI-TOF MS (Autoflex MALDI-TOF MS, Bruker Daltonics, Bremen, Germany). All the chemicals used were HPLC sequencing grade quality or higher. Peptide masses were searched from databases using three search engines: Mascot (http://www.matrixscience.com), ProteinProspector (http://prospector.ucsf.edu/), and Prowl (http://prowl.rockefeller.edu/). Oxidation of methionine, carbamidomethylation of cysteine, maximum of two missed cleavages, and mass tolerance of 100 ppm were allowed. The search results were evaluated by considering the probability score, sequence coverage, and correspondence between the estimated and the experimental p*I* and molecular weight. All selected protein spots were identified at least two times with high probability score.

Collagen Gel Contraction Assay (CGC)

The collagen solutions for contraction assays were prepared using rat-tail type I collagen (BD Biosciences, Bedford, MA, USA). The collagen solutions also contained the following components: 1× DMEM (Gibco, Paisley, Scotland), 10× DMEM (Gibco), 2 mM glutamate, and fetal calf serum (FCS, Biochrom AG, Berlin, Germany). The solutions were always prepared for four simultaneous contraction assays, and the assays were conducted in the wells of 24-well culture plates (Nunc, Roskilde, Denmark).

The collagen lattices for contraction assays were prepared in three phases. First, collagen mixture containing type I collagen at the final concentration of 0.5 mg/mL and 10 % FCS without the cells was added to the wells and allowed to gel at 37°C for 1 hour. Next, the collagen mixture above and collagen mixture containing the cells (12.5×10^3 CADASIL VSMCs or control VSMCs per gel) was added. After gelling, DMEM containing 10 % FCS was added. At the beginning of the contraction assays, the gels were gently detached from the walls of the wells to initiate cell-mediated collagen contraction at 37 ˚C in 95% air/5% CO_2. After 24 h the contracted collagen gels were fixed with 1% neutral-buffered formalin for 2–24 h at room temperature. The degree of contraction was assessed by measuring the area of the gels with an imaging densitometer (MCID Image Analyzer). The experiments were repeated three times, each with four replicates.

Data of the collagen gel contraction assays in Figure 2 are shown as a mean and standard deviation (SD). An unpaired Student *t* test was employed for comparing the groups of data. All *P*-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Characterization of the Cells

Three cell lines were derived from babies of CADASIL mothers and three other lines from babies of healthy non-CADASIL mothers. All these cell lines were tested for the presence of the C475T (R133C) mutation. The mutation was detected only in one cell line from a CADASIL mother´s baby. This cell line is referred in the text as CADASIL VSMCs and the other five cell lines as control VSMCs.

The CADASIL and control VSMCs were identified to be VSMCs by similar positive immunostaining with anti–smooth muscle β-actin antibody. The CADASIL VSMCs proliferated approximately 10 times slower than the control VSMCs. Further, the Notch3 expression of the cells was verified in Western blot analysis, where the CADASIL VSMC showed slightly elevated expression levels of full-length Notch3 and N3ECD compared with the control VSMC.

Expression Profile of the Proteins

Partial images of individual silver-stained 2-DE gels of CADASIL and control cell lysates are shown in Figure 1A. We detected in total 626 protein spots. A

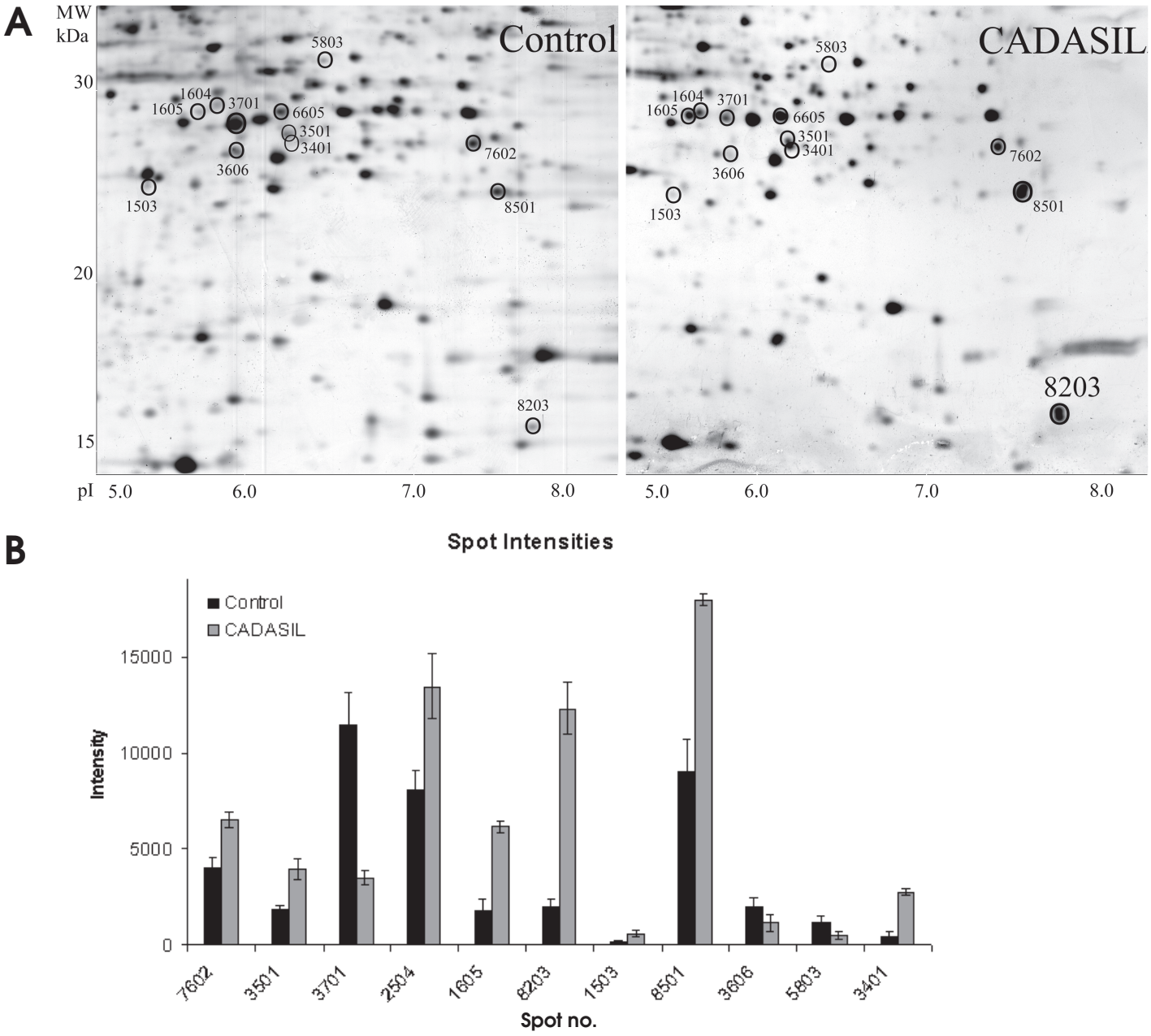

Figure 1. Two-dimensional (2-D) gel images of the CADASIL and control samples and the spot intensities from the selected spots. (A) The partial images of the silver stained 2-D gels of human CADASIL and control VSMCs. The targeted protein analysis revealed 11 spots with a statistically significant difference ($P < 0.05$) in spot intensity between CADASIL and control groups (3 replicates). (B) The silver stained 2-D gels were analyzed with a PDQuest analyzing program. The statistical significance of the differences was calculated using the Student *t* test and the one-way ANOVA. The spot intensities in the image are mean values of three replicates in the group and ± SEMs are shown in each bar.

targeted image analysis showed 35 spots with a statistically significant difference ($P < 0.05$) between CADASIL and control groups. The quality of the automated spot detection and matching analysis was visually verified. Nine of the spots were further selected for mass spectrometry analysis. An additional requirement of at least a (±) two-fold difference in the spot intensity was applied. In addition, two proteins with statistically significant difference but less than two-fold difference in the expression level were selected (Table 1 and Figure 1B). Eight of the selected spots were upregulated in the

Table 1. Identified Proteins[a]

Spot no.	Protein name	SwissProt Accession no.	Theoretical MW/pI	Sequence coverage	Probability score	ANOVA (P)	Change CADASIL/Control
Protein degradation and folding							
7602	Proteasome component C3	P25787	25.8/ 7.1	20%	64	< 0.0001	1.6
3501	Proteasome β chain	P28070	26.0/5.6	42.1%	69	< 0.02	2.1
3701	Ubiquitin carboxyl-terminal esterase L1	P09936	24.9/ 5.2	43%	100	< 0.0002	0.3
2504	Heat shock protein 27	P04792	22.8/6.0	39.5%	84	< 0.03	1.7
Cytoskeleton interaction/SMC contraction							
1605	Rho protein GDP-dissociation inhibitor	P52565	23.3/ 5.0	28%	90	< 0.000003	3.4
8203	Profilin 1	P07737	15.1/ 8.5	38%	111	< 0.004	6.3
1503	Cystein and glycine rich protein 1	P21291	21.3/ 8.9	64%	88	< 0.05	3.7
Free radical scavenger/cellular stress							
8501	Superoxide dismutase. mitochodrial	P04179	24.9/ 8.3	33%	82	< 0.00004	2.0
3606	Glutathione S-transferase P1	P09211	23.4/ 5.3	53%	101	< 0.05	0.5
5803	Glutathione S-transferase omega	P78417	27.8/ 6.2	20%	66	< 0.03	0.4
Other							
3401	Aspartyl-tRNA synthetase	P14868	57.5/6.1	14%	72	< 0.00002	5.7

The spot numbers are the same as in Figure 1. The accession number is Swiss-prot database accession number; theoretical molecular weight (MW) and isoelectric point (p*I*) for the proteins are obtained from Swiss-prot database. Probability scores and sequence coverage are from Mascot search engine, and probability scores >60 were considered as significant. The significance of the intensity differences was calculated with one-way ANOVA (*P*-values), and the change in the average spot intensity of the CADASIL sample is represented as comparison to the average spot intensity of the control sample (CADASIL/Control).

CADASIL VSMC and three were downregulated.

The 11 identified proteins (Table 1) could be classified as follows: four are involved in protein degradation and folding, four in interactions with cytoskeletal proteins and in smooth muscle cell contraction, three related to cellular stress, and one with unknown significance.

Biological Functions of the Identified Proteins and Their Possible Role in Cadasil

Protein degradation and folding. Four of the proteins were found to be involved in protein degradation and folding: Proteasome β chain (upregulated), proteasome component C3 (upregulated), small heat shock protein 27 (HSP27) (upregulated), and ubiquitin carboxyl-terminal esterase L1 (UCH-L1) (downregulated) (Table 1).

Proteasome β Chain and Proteasome Component C3. Proteasome β chain and component C3 are T1A family peptidases and part of the 20S core particle (14) of proteasome multicatalytic proteinase complex.

Newly synthesized proteins form disulfide bonds and gain their three-dimensional structure in the ER. Misfolded proteins are recognized by the quality control system in the ER and are dislocated through a protein-conducting channel, polyubiquitylated, and degraded by proteasome (15). Production of misfolded proteins stimulates the ER quality control system and causes ER stress, which leads to activation of the unfolded protein response (UPR), which in turn upregulates the expression of proteins needed for degradation (16). Upregulation of proteins in the 20S proteasome has been reported in conjunction with several misfolding disorders (17,18), thus supporting our notion that the mutation in the N3ECD causes misfolding of the protein (3), leading to activation of the proteasomal degradation pathway and possibly UPR. On the other hand, in HEK cells the mutated Notch3 was shown to reach the plasma membrane, although the trafficking of the protein was slower compared with wild-type Notch3 (8), suggesting a leakage of the quality control in ER. This leakage would also explain the accumulation of mutated N3ECD (and GOM) on the surface of VSMCs as occurs in CADASIL.

HSP27. HSP27 is a stress-inducible small heat shock protein with molecular chaperone activity. Molecular chaperones bind partially denatured proteins, thereby preventing irreversible protein aggregation during stress. HSP27 is shown to protect cells from α-synuclein–induced toxicity and aggregation (23) and β-amyloid aggregation (24). The mutated Notch3 is inclined to misfold, and it has been shown to aggregate out side of the cell as a major component of the GOM (25). In the CADASIL VSMCs the upregulated HSP27, as a chaperone protein, assists mutated Notch3 to fold and to make it less prone to aggregation. HSP27 is also involved in SMC contraction, and its role in actin reorganization is discussed in more detail in the following section on "RhoGDI, HSP27, and Profilin 1."

UCH-L1. UCH-L1 as a dimer has ubiquitin-ligase activity, but as a monomer it functions as a deubiquitylating enzyme (19,20). UCH-L1 conjugates

proteins to ubiquitin through lysine 63 linkage (20), which has been implicated in endocytosis (21).

Expression of plasma membrane receptors is regulated by ubiquitylation, which directs the receptors for internalization and lysosomal degradation or recycling to plasma membrane (21). On the other hand, a mutation that impairs the ubiquitin ligase activity of ubiquitin ligase Cbl blocks epidermal growth factor receptor degradation and directs the receptor from the sorting endosome to the recycling pathway (22). It is tempting to speculate that the UCH-L1 might be involved in a similar regulation as ubiquitin ligase Cbl, marking the plasma membrane receptor with ubiquitin for internalization, and at the same time sorting the cargo for degradation, or if subsequently deubiquitylated, recycled to the plasma membrane. Misfolding of N3ECD could cause a failure in the recognition of Notch3 by UCH-L1. This impairs either internalization or sorting in endosome of misfolded Notch3, which therefore either remains attached or becomes recycled to the plasma membrane, leading to its accumulation on the VSMC surface (1).

Smooth muscle cell contraction. Four of the identified proteins, Rho protein GDP-dissociation inhibitor (RhoGDI) (upregulated), HSP27 (upregulated), profilin 1 (upregulated), and cysteine and glycine rich protein 1 (CRP1) (upregulated) interact with cytoskeletal proteins and are involved in smooth muscle cell contraction.

At the early stage of CADASIL, before the destruction of VSMCs has occurred, the VSMCs of small arteries appear contracted. In ophthalmological examinations the diameters of retinal arterioles are smaller than in the age-matched controls, and they appear similarly contracted as in hypertensive patients (26). This conforms to the reduction of blood flow in the peripapillary retina demonstrated by scanning laser Doppler flowmetry (27). Furthermore, the reduced cerebral blood flow in CADASIL is associated with increased distal cerebrovascular resistance (28) and it can be decreased by administration of acetazolamide (29) or carbon dioxide (28, 30). In addition, it was recently shown that CADASIL patients have enhanced maximal constriction response to angiotensin II in the resistance arteries (31).

RhoGDI, HSP27, and profilin 1. RhoGDI is a cytosolic regulator of Rho family GTPases (32-35), and the amount of RhoGDI in a cell is similar to the sum of Rho family GTPases (36). Therefore, the upregulation of RhoGDI indicates upregulation of the Rho family GTPases and enhanced signaling of the Rho family GTPase-regulated pathways, including VSMC contraction. Rho family GTPases can be activated by angiotensin II, the hypertension-inducing effector molecule in the renin-angiotensin system involved in the regulation of blood pressure (37,38). Rho family GTPases activate Rho kinases (39) and a signaling cascade which leads to increased microtubule phosphorylation and facilitation of microtubule disassembly, actin filament assembly, and finally, promotion of smooth muscle contraction (40-42).

In addition to protecting cells from protein aggregation, HSP27 also regulates reorganization of actin filaments. Angiotensin II is shown to induce HSP27 phosphorylation through AT1-receptor activation (43). Phosphorylated HSP27 stabilizes actin filaments and plays a central role in SMC contraction. HSP27 interacts with tropomyosin and with signaling proteins ROCK-II and RhoA in agonist-induced contraction of SMC (44–46).

The most markedly upregulated protein in our analyses was profilin 1, which is a regulatory protein of actin organization and necessary for actin polymerization during contraction of smooth muscle cells (47,48). Profilin 1 binds to unpolymerized globular actin, promotes actin polymerization (47) and enhances Rho family GTPase–mediated actin-filament assembly (49–51). Profilin interacts with a large number of proline-rich proteins, for example, with Rho family GTPases activated proteins, which are involved in endocytosis and vesicle trafficking (51-53).

CRP1. CRP family members have been shown to interact with two filamentous actin binding proteins, α-actinin (54), and zyxin (55), and CRP1 is specifically expressed in smooth muscle cells (56).

Free-radical scavenging/cellular stress. Three proteins, mitochodrial manganese superoxide dismutase (MnSOD) (upregulated), glutathione S-transferase P1 (GST P1) (downregulated), and glutathione S-transferase omega (GST omega) (downregulated), were identified as proteins protecting cells from various toxic compounds. MnSOD converts free radicals to oxygen and hydrogen peroxide. Upregulation of MnSOD expression is induced by ROS (57). The activity of SOD has been shown to help in protecting cells from free-radical damage, protein denaturation, and many other forms of progressive cell degradation (58).

GST P1 and GST omega are cytosolic enzymes, which are involved in detoxification of a wide range of compounds, including carcinogens, therapeutic drugs, and products of oxidative stress by glutathione. In addition, GSTs are involved in degradation of amino acids and synthesis of steroid hormones and important metabolites of arachidonic acid and therefore they have influence on many signaling pathways of the cell (59).

ER-associated degradation eliminates unfolded proteins under normal conditions. When stress is applied, for example by accumulation of a misfolded protein, the UPR is required to increase ER-associated degradation (60). During the formation of disulfide bonds, electrons from thiol groups of substrate proteins are transferred to molecular oxygen by UPR-regulated ER oxidative folding machinery, with generation of ROS as a byproduct in ER as well as in mitochondria (61,62). It was suggested that improperly formed sulfur bridges are reduced by glutathione, allowing sulfur groups to interact again with the proteins from the oxidative folding machinery. If mutated proteins permanently fail to form correct sulfur bridges, a futile cycling between disulfide bonding and breakage would continue with each cycle

activating UPR, generating ROS and consuming glutathione. This process can lead to depletion of glutathione and further diminished ability of cells to eliminate increasingly produced ROS (62,63).

In CADASIL VSMCs, where the mutated Notch3 fails to produce correct sulfur bridges, the downregulation of GSTs could be a consequence of depletion of cytosolic glutathione as a result of repeated cycles of thiol group reduction and oxidation. HSP27 is shown to protect cells from various stress factors and therefore it may also have a function in misfolding-induced oxidative stress. Overexpression of HSP27 increases the levels of glutathione by an unkown mechanism (64) and it inhibits cytocrome c–dependent activation of procaspase 9(65) and therefore prevents apoptosis. The upregulation of MnSOD indicates increased mitochondrial ROS production, ER stress, and UPR activation (57,62). This is consistent with the observations that glutathione depletion resulted in subsequent mitochondrial dysfunction and ROS accumulation (66) and also induced growth inhibition (62). Our findings that mtDNA mutations are more frequent in CADASIL patients (67) and the CADASIL VSMCs used in this study have impaired growth capacity conform to the observations above.

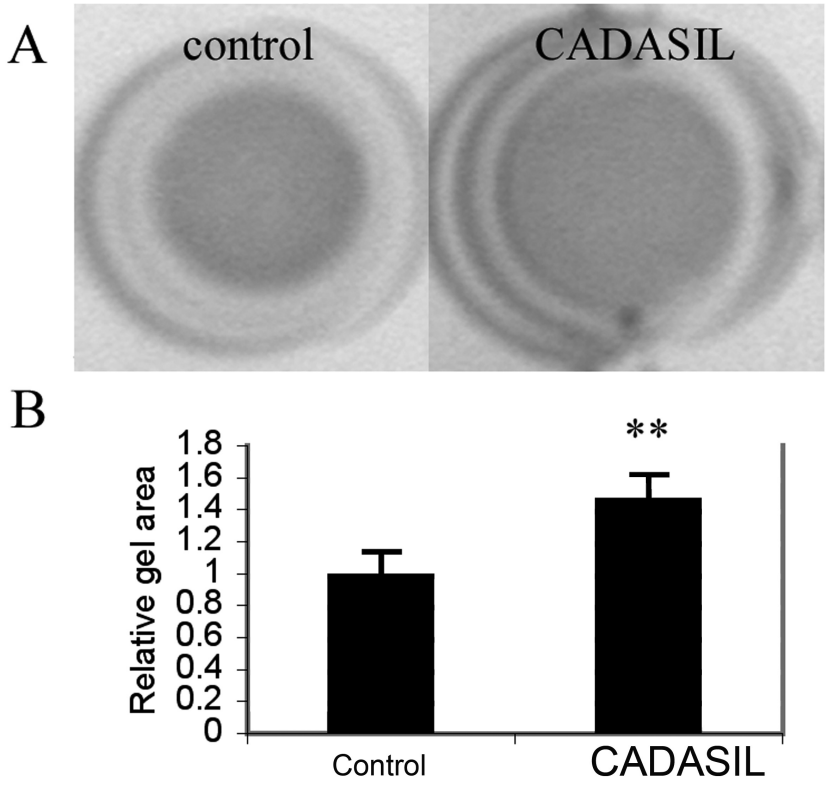

Figure 2. Collagen gel contraction (CGC) by control and CADASIL VSMCs. (A) Representative images of contracted collagen gels by control and CADASIL VSMCs. The gels contained 12.5 × 10^3 cells/well and were cultured for 24 h before assessing gel contraction. Control VSMCs contracted spontaneously collagen gel markedly more than CADASIL VSMCs. (B) Quantification of CGC by control and CADASIL VSMCs. Columns indicate the mean of 4 replicated assays and capped bars indicate standard deviation (SD). Statistically significant difference between normal and CADASIL VSMCs is indicated by asterisk (**$P < 0.01$, unpaired Student t test).

Collagen Gel Contraction Assay

Because CADASIL cells have substantial upregulation of several actin-interacting and SMC contraction-involved proteins, we used a CGC assay to analyze the ability of these cells to contract collagen-rich gels. The results demonstrated that the relative collagen gel area is larger in the CADASIL cell culture than in the control cell culture, indicating that the ability of the CADASIL cells to spontaneously contract collagen gels is markedly diminished compared with the control cells ($P < 0.01$) (Figure 2). Because in this CGC assay the cells are not exposed to any contraction agonist, this finding emphasizes the importance of additional factors normally present in vivo such as angiotensin II or noradrenaline (31).

INTERRELATIONSHIP OF OUR FINDINGS

We have a unique material of genetically genuine cultured human CADASIL VSMCs, which is to our knowledge the only CADASIL cell line available. Our results are supported by other studies on CADASIL patients and Notch3 transfected cell lines.

CADASIL is caused by mutations in N3ECD, which lead to impaired disulfide bridging and hence altered conformation of Notch3 (3). The fact that Notch3 signaling is affected only by those mutations located in the ligand binding site indicates that inhibition of the Notch3 signaling due to mutations in N3ECD is not the direct cause of the disease (8–10). The insidious onset of the disease implicates secondary, delayed impairment of the signaling, which is most likely related the pathognomonic accumulation and possible impaired degradation of N3ECD and GOM.

Our proteome analysis of CADASIL and control VSMCs revealed several alterations which suggest that the CADASIL VSMCs attempt to correct the improper sulfur bridging that leads to protein misfolding. The consequent ER stress and activation of UPR leads to increased ROS formation with subsequent protective attempts by upregulating MnSOD and resultant glutathione depletion and inhibition of growth (16,60,62). The above-described UPR does not, however, prevent trafficking of misfolded Notch3 molecules to the cell surface, though it has been reported to be slowed

down (8). The accumulation of N3ECD on the surface of VSMCs could be further intensified by a failure in recognition of mutated N3ECD by UCH-L1, leading to impaired internalization or recycling of the receptor. Finally, this accumulation of misfolded N3ECD would lead to gradual impairment of the signaling and, consequently, insidious onset of the disease.

The arteries of CADASIL patients are contracted (27,29,70,71), and they have increased maximum response to angiotensin II, suggesting "a selective systemic microvascular vasoconstrictor abnormality in angiotensin II pathway" (31). In our proteomic analysis, several of the alterations in protein expression in CADASIL VSMCs were involved in regulation of VSMC contraction, among them RhoGDI, which plays a regulatory role in Rho family GTPase signaling (41), as well as downstream proteins in this pathway. In addition, in the CGC assay CADASIL VSMCs possessed altered spontaneous contractility compared with control VSMCs. These findings are intriguing, because angiotensin II stimulates smooth muscle cell contraction by a Rho family GTPase-dependent pathway (72–75). Because angiotensin II downregulates Notch3 expression in VSMCs (76), the accumulation of N3ECD on the surface of CADASIL VSMCs could activate a feedback loop and enhance the ability of the VSMCs to respond to angiotensin II stimulus, and as a "side effect" lead to accentuated contraction of VSMCs demonstrated in CADASIL patients (26,27). Angiotensin II has also been shown to stimulate type I collagen expression via activation of transforming growth factor β signaling (77), which conforms to the marked fibrosis of cerebral small arteries in CADASIL. The possibility that angiotensin II signaling pathway may be a therapeutic target to prevent contraction and stenosis of arteries in CADASIL patients is strengthened by the findings of this study.

In the present study we have compared proteomic expression of cultured genetically genuine CADASIL and control VSMCs isolated from human umbilical cord. Several proteins that were up- or downregulated were related on one hand to control mechanisms and consequences of protein misfolding—in CADASIL misfolding of the mutated Notch3—and on the other hand to VSMC contraction. According to current knowledge there is an accumulation of the non–S1-cleaved (full length) mutated Notch3 as well as S1- and S2-cleaved mutated N3ECD. Production of misfolded Notch3 leads to ER stress and UPR and secondarily to VSMC contraction. Our current hypothesis is that CADASIL is caused by a persistent ER stress and/or impaired internalization and degradation of mutated misfolded Notch3 molecules, which leads to derangement of the Notch3 signaling cascade and to subsequent degeneration of VSMCs and overproduction of collagen type I. These processes eventually lead to fibrosis and vascular stenosis and consequent ischemic lesions as seen in CADASIL patients.

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