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Notch3 protein expression in skin fibroblasts from CADASIL patients

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ABSTRACT

Aim: CADASIL is an inherited cerebrovascular disease caused by mutations in the *NOTCH3* gene. Notch signaling is involved in a broad spectrum of function, from the cell proliferation to apoptosis. Thus far, because the molecular mechanism underlying the pathological alterations remains unclear and taking into account that fibroblasts contribute to the integrity of the vasculature, our aims was to establish whether fibroblasts, in subjects carrying different *NOTCH3* mutations, show abnormalities in the protein expression.

Methods: We performed the investigation on skin fibroblasts in culture obtained from three CADASIL patients and normal subjects. The patients were genetically characterized, and carried a p.R61W, a p.C174T, and p.R103X, mutation respectively. Notch3 expression was first evaluated on fibroblasts by immunofluorescence analysis, then western blot on cellular extract was utilized to validate the immunofluorescence results.

Results: The Notch3 immunoreactivity was clearly detected along the cellular body and in the cellular nuclei of the control fibroblasts. We observed a marked, statistically significant, reduction of the fluorescence immunoreactivity in the fibroblasts from patient with the classical C174T cysteine mutation and a less pronounced reduction in the other two subject's samples with respect to the normal controls. These data were confirmed by the immunoblot analysis.

Conclusions: Our results show that the investigated three *NOTCH3* mutations are associated with a reduction of the levels of Notch3 expression in vitro. Because the smooth muscle cells appear to be predominantly involved in this cerebrovascular disease, our result, despite the limitation of the sample size examined, clearly suggest that also fibroblasts, directly involved in making the vascular basal lamina and in maintaining the vascular integrity, may play an important role in the mechanism responsible for the disease.

1. Introduction

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL; OMIM 125310) is an inherited arteriopathy characterized by recurrent subcortical ischemic strokes, migraine with aura, cognitive decline and vascular dementia [1,2]. It results from mutations in the *NOTCH3* gene, consisting of 33 exons spanning roughly 7 kb and encoding a single pass transmembrane heterodimer receptor protein Notch3 (N3) involved in cell signaling and cell differentiation [3]. More specifically N3, comprising an N-terminal extracellular domain (NECD), a transmembrane domain (NTMD) and an intracellular domain (NICD) [4], is synthesized as single precursor protein which is cleaved during transport to the cell surface (S1 cleavage), where it is expressed as heterodimer. Upon binding of its ligand (a protein of the Delta/Jagged family), N3 receptor undergoes two other proteolytic cleavages (S2 and S3 cleavage), mutually dependent: these events generate the NICD fragment, which released from the NTMD enters the nucleus for activating the transcription of its target genes [5].

1.1. CADASIL pathogenesis

Pathogenic mutations in CADASIL occur in exons 2-24, encoding for the NECD, and usually they involve the introduction or elimination of a cysteine residue within one of the 34 EGF-like repeats of the protein, leading to an odd number of cysteine residues. Although currently it is

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unknown how CADASIL mutations trigger the disease, a common explanation could be provided taking in account that an odd number of cysteine residues is predicted to impair normal disulphide pairing within the N3 ectodomain [6]: consequently, this impairment may induce protein misfolding, causing aberrant interaction between the N3 receptor and its ligand [7] and/or an altered protein maturation, targeting and degradation. This suggestion would be in agreement with the evidence of the accumulation, aggregation and deposition of the NECD at the plasma membrane of vascular smooth muscle cells (VSMC) within both cerebral and extra-cerebral vessels of patients [8]. Despite a great majority of CADASIL-causing mutations are missense, other rare types of mutations such as duplications, small in-frame deletions, splice site mutations and a deletion/insertion have been detected (www.hgmd.cf.ac.uk/) [9–12].

In addition to the mutations that alter the number of cysteine residues in N3, several cysteine-sparing mutations have been also described both in patients with a CADASIL-archetypical clinical and imaging phenotype and patients with different MRI findings (such as a less frequent involvement of the anterior temporal lobes), late disease onset and a slower clinical course, leading to the suggestion that they might be responsible for milder CADASIL phenotype. However, the debate on their role in causing CADASIL phenotype is still questioned: there is no clear evidence whether these variants are causative of CADASIL, whether they are associated with another form of cerebral small vessel disease (SVD), or whether they are not pathogenic and therefore represent rare single-nucleotide polymorphisms occurring in the general population [9,11,13–21].

In order to identify the molecular mechanisms underlying the disease, a lot of investigation focused on the study of the N3 function and traffiking in mouse models and/or in cells transfected with *NOTCH3* gene carrying specific mutations, have already been carried out [22–27].

On the other hand, at date, few data concerning the cellular N3 expression, both in healthy and CADASIL patients, are available. Even though Di Maio and coll. [28], by using Western Blotting (WB), have highlighted the N3 protein expression both in human lymphocytes and skin fibroblasts, no data regarding the cellular expression and localization of the N3 protein, in CADASIL patients, immunoreactively detectable, have been reported. Since fibroblasts are involved in the maintenance of the vascular integrity [29] and whereas the cellular expression of a particular protein is the result of a set of biomolecular events including synthesis, maturation, dislocation and function, we aimed to establish whether fibroblasts, in subjects carrying different *NOTCH3* mutations, show abnormalities in the protein expression and location. Due to this approach, despite the limitation of the sample size examinated, we could obtain potential claims of new cellular and molecular aspects, need to be considered in CADASIL.

2. Subjescts and methods

2.1. Patients

We performed the investigation on skin fibroblasts in culture from three CADASIL patients with comparable clinical manifestations of the disease (see Table 1) and two normal subjects. The patients, designated as CAD1, CAD2 and CAD3, were genetically characterized, and carried, respectively, a p.R61W (EGF-like domain 1), p.C174T (EGF-like domain 4), and p.R103X (EGF-like domain 2). The mutation in the CAD2 patient was a classical cysteine involving mutation, while the other two mutations, in CAD1 and CAD3 patients, were two cysteine sparing mutations. The mutation R61W is a missense mutation at position 61 of the protein falling in the EGF-like domain 1 described at first by Brass and coll. [30]. The p. R103X (c.307 C/T) mutation causes an introduction of a stop codon at 103 position. The formation of such premature stop codon results in the production of a truncated protein product lacking part of exon 3 and all the subsequent exons (4/33); therefore, it is characterized by the absence of all EGF-like repeat domains starting at n2 except EGF-like [31].

2.2. Skin fibroblast collection and culture

Primary skin fibroblasts were obtained by explants from skin punch biopsy [32] after informed consent. The biopsies were processed simultaneously to establish primary cell lines. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 125 μg/ml streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed on cells with passage numbers ranging from 5 to 15, to avoid artefacts due to senescence.

2.3. Immunofluorescence microscopy

Cells were cultured for 48 h on 10 mm square coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 in TBS and blocked in 10% BSA in TBS. Fixed fibroblasts were then treated with a mouse anti Human Notch3 antibody that recognizes the extracellular domain NECD (domain 1,2,3) (Abnova, CA, USA) and a rabbit antibody that recognizes the intracellular domain NICD (Santa Cruz, CA, USA). Alexa Fluor 488 and Alexa Fluor 546 conjugate specie specific secondary antibodies were used to reveal the immunocomplexes (Invitrogen, CA, USA). A second rabbit anti NICD antibody (Novus Biological, CO, USA) and an anti NECD, recognizing the domain 19,20,21,22 (Novus Biological, CO, USA) were also utilized to confirm data from the first one. Each antibody was employed at the dilution of 1:200. Images were captured using a conventional fluorescence microscope Nikon's Microphot–FX (Nikon, Tokyo, Japan) equipped with a Nikon D40 reflex camera and analyzed using NIH ImageJ browser software.

2.4. Cellular lysis

Cells at 60% confluence in 6 cm diameter dishes were washed with sterile PBS and successively manually scraped in fresh PBS containing protease inhibitor (0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 1 mM PMSF, and 0.2 mM sodium orthovanadate). The cellular suspension was centrifuged at 3.000 RPM for 10 min and the recovered cell pellet was treated with Laemmli lysis buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 3 min in order to obtain a whole cellular extract.

2.5. Western blot analysis

Sample protein extract was subjected to protein quantitation by using the Bio-Rad Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction, and bovine serum albumin as standard. 20 μg of protein were loaded and resolved on 8%, Tris-HCl SDS-PAGE gel with prestained molecular weight marker (Sigma 7B2) and successively blotted by using a semidry apparatus, Trans-blot SD (Bio-Rad Laboratories, Hercules, CA, USA) on nitrocellulose membranes (Bio-Rad). The membranes were blocked in 1% BSA in TBS and incubated with the same primary antibodies utilized for the immunofluorescence at dilution of 1:2.000. Specie specific secondary antibodies HRP conjugate (Santa Cruz, CA, USA) at 1:10.000 dilution, chemiluminescence substrate (Merk-Millipore KGaA, Darmstadt, Germany) and X-ray film CL-XPsure thermo (Thermo Scientific, Rockford, IL, USA) were employed to reveal the immunocomplexes.

2.6. Statistical analysis

Statistical significance of raw data among groups was evaluated using unpaired Student's *t*-test. Results were considered significant for $P < 0.05$. and data are presented as mean ± standard deviation. Data