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Processing of chromogranins/secretogranin in patients with diabetic retinopathy

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ABSTRACT

Aims: Inflammation has been linked to the development of diabetic retinopathy (DR). Chromogranins A, B (CgA, CgB) and secretogranin II (SgII), are prohormones overexpressed in inflammatory diseases. The present study was conducted to evaluate the presence and processing of these prohormones in the vitreous of patients with DR (DV), compared with nondiabetic vitreous (NDV).
Methods: Thirteen DV and 14 NDV samples were collected during vitreoretinal surgery. ELISA, Western blot, RP-HPLC, dot blot, protein sequencing and mass spectrometry were used to study the quantitative expression and the processing of CgA, CgB and SgII.
Results: CgA, CgB and SgII presence was higher in DV than in NDV. Mean concentration of CgA evaluated by ELISA was 90.8 (±90.1) n L^{-1} in DV vs. 29.7 (±20.9) in NDV ($p = 0.039$). In NDV, Western blot indicated that only short CgB-derived peptides were identified. In DV, proteomic analyses showed that long CgA-, CgB- and SgII-derived fragments and α1-antitrypsin were overexpressed, suggesting possible inhibition of the proteolytic process.
Conclusions: This study shows differences in the presence and endogenous processing of CgA, CgB and SgII from DV vs. NDV. In DV, the increase of complete granins and the attenuation of their endogenous proteolytic processing could participate in DR progression by reducing the presence of regulatory peptides, important for the pro-/anti-angiogenic balance in the eye.

1. Introduction

Diabetic retinopathy (DR) is one of the major causes of legal blindness in people under 50 years of age in the US and Europe [1]. In DR, retinal ischemia consecutive to capillary occlusion leads to a vitreoretinal neovascular proliferation, which is due to overexpression of proangiogenic factors, such as vascular endothelial growth factor (VEGF) [2]. The close relationship between vitreous and the vitreoretinal proliferative process is clearly established. Numerous studies have established that chronic inflammation occurs in diabetic retinal tissue during proliferative DR, but the molecular mechanisms leading to the complex inflammatory response in DR, with the involvement of polymorphonuclear neutrophils (PMNs) [3,4], cytokines and neuropeptides [5–7], is still not sufficiently understood. Chromogranin A, chromogranin B (CgA, CgB) and secretogranin II

Abbreviations: A1AT, alpha1-antitrypsin; CgA, chromogranin A; CgB, chromogranin B; DR, diabetic retinopathy; DV, diabetic vitreous; HbA1C, glycated haemoglobin; HRB, hematoretinal barrier; nanoLC-MS/MS, nano-liquid chromatography–mass spectrometry/mass spectrometry; NDV, nondiabetic vitreous; PC, prohormone convertase; PDR, proliferative diabetic retinopathy; Mild NPDR, mild non proliferative diabetic retinopathy; Moderate NPDR, moderate non proliferative diabetic retinopathy; PMN, polymorphonuclear neutrophils; OHA, oral hypoglycaemic agents; PRP, panretinal photocoagulation; PVDF, polyvinylidene difluoride membrane; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SgII, secretogranin II; SN, secretoneurin; TMB, tetramethyl benzidine; VEGF, vascular endothelial growth factor.

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(SgII) are ubiquitous acidic glycophosphoproteins present in the nerve, endocrine and immune systems [8] and related to inflammatory processes [9,10]. Endogenous expression and processing of these prohormones are tissue-dependent. This processing leads to multifunctional biologically active derived peptides [11]. Among these, pancreastatin, a CgA-derived peptide, inhibits the release of insulin from pancreatic beta cells [12] and is involved in the metabolism of glucose [13], while WE14, another CgA-derived peptide present and generated in ocular nerve tissues [14], has been recognized as an antigen for diabetogenic CD4 T cells in a diabetic mouse model [15]. Furthermore, secretoneurin (SN), a SgII-derived peptide, modulates neutrophil migration [16] and acts as a proangiogenic cytokine [17]; two other CgA-derived peptides, chromofungin and catestatin are able to stimulate polymorphonuclear neutrophils PMNs [18]. Previous studies have demonstrated the presence of granins and their derived peptides in ocular fluids [19]. However, the vitreous presence of CgA and CgB, the two predominant granins in humans, remains largely unknown.

In order to determine whether a relation exists between granins and DR, the presence of CgA, CgB and SgII and their derived peptides was analyzed in the vitreous of diabetic patients and compared with the vitreous of nondiabetic patients using several proteomic approaches.

2. Research design and methods

2.1. Human vitreous humor sample preparation

Diabetic vitreous (DV) samples ($n=13$) and nondiabetic vitreous (NDV) samples ($n=14$) were obtained from patients undergoing vitrectomy surgery from January 2007 to December 2009, in the Nouvel Hôpital Civil Ophthalmology Department in Strasbourg, France. Institutional review board or ethics committee approval was not required for this study, which conformed to European and French legislation. Informed consent was obtained from all patients and the study adhered to the tenets of the Declaration of Helsinki. DV samples were collected from 13 diabetic patients (9 men and 4 women) with DR and NDV samples from 14 nondiabetic patients (5 men and 9 women) with idiopathic epiretinal membranes. The mean age of the patients was comparable in the diabetic and non diabetic groups (67 vs. 71 years respectively, $p \geq 0.05$, Mann–Whitney Test). Characteristics and treatments of the diabetic patients are reported in Table 1. Patients undergoing combined cataract and vitreoretinal surgery were excluded from the study. For each sample, acidic protein was extracted with 0.1% (v/v) trifluoroacetic acid in milliQ water. After centrifugation at 12,500 rpm for 15 min at 4 °C, the resulting supernatants were used for experiments. Total protein concentration was estimated using the Bradford assay (Protein assay; Bio-Rad, Marnes-la-Coquette, France). Vitreous samples were aliquoted and stored frozen at −20 °C until assay.

2.2. CgA concentration by ELISA immunoassay

The concentration of CgA was evaluated by a sandwich ELISA-type immunoassay (Chromoa, Cisbio Bioassays, Bagnols/Cèze, France). A first monoclonal antibody raised against human recombinant CgA was immobilized on the microplate and captured the CgA proteins contained in the calibrators (human recombinant CgA) and samples. After a 2 h incubation at room temperature, the microplate was washed and the fixed proteins were then recognized by a second monoclonal antibody conjugated to HRP (horseradish peroxidase). After a second incubation for 2 h at room temperature, the unfixed reagents were eliminated by washing. The colorimetric reaction was started by the addition of an HRP substrate, TMB (3, 3′, 5, 5′ tetramethyl benzidine). After the reaction was stopped, the optical density (OD) of each well was read at 450 nm. The OD values measured were proportional to the CgA protein concentration contained in the calibrators and samples. Intra- and inter-assay precision errors were lower than 6 and 12%, respectively.

2.3. Western blot analysis

Soluble proteins from vitreous samples (20 µg) were separated by SDS-PAGE (12% polyacrylamide gel) electrophoresis and electrotransferred (45 min, 75 V) on a polyvinylidene difluoride membrane (PVDF) (Amersham Biosciences, Bucks, UK). CgA, CgB and SgII immunodetection was evaluated using specific rabbit polyclonal antibodies. The epitopes of the antibodies corresponded to the bovine fragments anti-bCgA$_{4\text{-}16}$ (dilution, 1:6,000), anti-bCgB$_{547\text{–}560}$ and anti-bCgB$_{614\text{–}626}$ (dilution, 1:2,000) [20] and anti-bSgII$_{165\text{-}182}$ (dilution, 1:3,000) [21]. Secondary antibodies were goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP, dilution, 1:30,000) (Sigma Aldrich, St. Louis, MO, USA). All the immunodetection tests were performed with the SuperSignal^{R} West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA). The apparent molecular mass of immunodetected bands was determined by comparison with molecular weight standards (Bio-Rad, Marne-la-Coquette, France).

Table 1
Characteristics and treatments of the diabetic patients studied.

Diabetic patient	Type of diabetes	Age	Gender	HbA1C (%)	Stage of DR	Previous laser treatment	Anti-diabetic treatment
1	Type 1	40	Male	9.80	PDR	PRP	Insulin
2	Type 1	47	Female	11.20	PDR	PRP	Insulin
3	Type 1	53	Male	8.30	PDR	None	Insulin
4	Type 1	54	Female	8.30	PDR	PRP	Insulin
5	Type 2	60	Male	7.30	PDR	PRP	Insulin
6	Type 1	61	Male	9.10	PDR	PRP	OHA
7	Type 2	64	Male	14.30	PDR	PRP	Insulin
8	Type 2	75	Male	8.50	Mild NPDR	None	OHA
9	Type 2	76	Male	6.90	Moderate NPDR	None	OHA
10	Type 2	77	Male	6.80	Mild NPDR	None	OHA
11	Type 2	85	Female	7.00	Mild NPDR	None	OHA
12	Type 1	85	Female	6.70	PDR	PRP	Insulin
13	Type 2	90	Male	12.00	Mild NPDR	None	Insulin

Mild NPDR : characterized by microaneurisms only.
Moderate NPDR: characterized by microaneurisms, and/or less than 20 intraretinal hemorrhages in each of the four fundus quadrants, and/or venous beading in less than 2 quadrants, and/or a few Intraretinal Microvascular abnormalities in one quadrant.
Mild and moderate NPDR and PDR are DR severity levels referring to the International Clinical Diabetic Retinopathy Disease Severity Scale (American Academy of Ophthalmology, Preferred Practice Patterns, 2003).
PRP: panretinal photocoagulation.
OHA: oral hypoglycaemic agents.