Elevated neprilysin activity in vitreous of patients with proliferative diabetic retinopathy

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Purpose: Diabetic retinopathy (DR) is the leading cause of blindness in the industrialized world. Hyperglycemia induces retinal hypoxia, which upregulates a range of vasoactive factors that may lead to macular edema and/or angiogenesis, and hence potentially to sight-threatening retinopathy. The control of signal-peptide activity by cell-surface proteases is one of the main factors regulating the development and behavior of organisms. In mammals, neprilysin is known to play a key role in these processes, and its inactivation can initiate cellular disorganization. Neprilysin is a rate-limiting peptidase involved in the physiological degradation of amyloid β (Aβ) in the brain. In this study, we measured both the enzymatic activity of neprilysin and the concentration of Aβ in patients with proliferative DR (as compared to their levels in patients with macular hole), and we analyzed their association.

Methods: In vitreous samples collected from patients who underwent vitrectomy, an HPLC-fluorometric system (recently established by us), and sensitive and specific enzyme-linked immunosorbent assays were used to determine the enzymatic activity of neprilysin and the concentration of Aβ.

Results: By comparison with the levels in the control (macular-hole) patients, there was a significant increase in neprilysin activity level and a significant decrease in Aβ level in proliferative DR patients. There was a significant inverse correlation between neprilysin and Aβ among all subjects.

Conclusions: Neprilysin activity and Aβ concentrations displayed converse changes in patients with proliferative DR.

Diabetic retinopathy (DR) is the leading cause of acquired blindness among young adults, and studies have shown that it is the hyperglycemia itself that initiates its development [1]. In the pathogenesis of retinopathy in diabetes, certain cells (retinal microvascular endothelial, Müller, and ganglion cells and pericytes) are lost selectively via apoptosis before any other histopathology is detectable and before any loss of vision is evident [2,3].

Alzheimer’s disease (AD) is characterized by the accumulation of amyloid β peptide (Aβ) in the brain. Aβ is constitutively produced by proteolysis of β-amyloid precursor protein (APP) [4,5]. The pathological formation of amyloid plaques is thought to be the primary force driving the pathogenesis of Alzheimer’s disease. Hence, much attention has been focused on the production of Aβ. Neprilysin (variously known as neutrophil cluster-differentiation antigen 10 (CD10), neuronal endopeptidase 24-11, or enkephalinase) is considered the most important enzyme for Aβ degradation in the brain [6,7]. Recently, genetic approaches using neprilysin-deficient mice have demonstrated the ability of neprilysin to cleave endogenous Aβ [8,9]. Moreover, a decline in neprilysin levels has been found in the brain in patients with early-stage sporadic AD [10], suggesting critical roles for reduced neprilysin activity in the incipient process of Aβ accumulation.

The prevalence of both Alzheimer disease and type 2 diabetes increases with age, and both have genetic components [11-16], raising the possibility that patients with Alzheimer disease may be more vulnerable to type 2 diabetes and raising the possibility of a linkage between the processes responsible for the loss of brain cells and the loss of β-cells in these diseases [17]. It has been reported that diabetes-related mitochondrial dysfunction is exacerbated by aging and/or by the presence or neurotoxic agents, such as Aβ, suggesting that diabetes and aging are risk factors for the neurodegeneration induced by these peptides [18]. Taken together, these data indicate that a correlation may exist among the age-related pathologies diabetes and AD. We have reported changes in the levels of Alzheimer’s disease-related factors (such as Aβ and Tau) in vitreous bodies obtained from patients with DR, indicating that Aβ42 and Tau may be implicated in the pathogenesis of DR and that the neurodegeneration processes of DR may share, at least in part, common mechanism with Alzheimer’s disease [19]. However, the detailed mechanisms are unclear at this time. Hence, the purpose of the present study was to determine the vitreous concentrations of neprilysin and Aβ in proliferative DR and to analyze the relationship between neprilysin and Aβ.
METHODS

Sampling from patients: This study was conducted according to the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients who underwent vitrectomy, and the study was carried out in accordance with the guidelines of the human studies committee of Kumamoto University. Patient diagnoses were idiopathic macular hole and proliferative DR (Table 1). Samples obtained from patients with a vitreous hemorrhage were not included in this study. The control group was made up of samples from eight eyes in eight patients with macular hemorrhage. Vitreous samples were also collected from ten eyes in ten patients with proliferative DR.

Measurement of neprilysin activity: Dansyl-D-Ala-Gly-p-nitro-Phe-Gly [20], Dansyl-D-Ala-Gly and thiorphan [21,22] were purchased from Sigma (St. Louis, MO). Recombinant human neprilysin was obtained from R&D Systems (Minneapolis, MN). Measurement of neprilysin activity was performed according to our previous report [23], with some modifications. In brief, 5 ml of vitreous fluid was added to 50 µl of 20 mM HEPES buffer (pH 7.2) containing a fluorescent substrate, Dansyl-D-Ala-Gly-p-nitro-Phe-Gly (200 µM), in the presence or absence of thiorphan (50 mM). Then, each sample was incubated for 60 min at 37 °C. After incubation for 5 min at 90 °C and centrifugation at 15,000 rpm for 5 min, an aliquot of the supernatant (10 µl) was used for HPLC analysis. The Dansyl-D-Ala-Gly produced from the fluorescent substrate was measured flurometrically using an HPLC apparatus (Shimazu, RF10AXL). A reverse-phase gel column (i.e., an Inertosil-3 C18 column; 4.6 mm Φ 400 mm; GL Science) with a guard column was used for the assay. The sample was eluted using 40% (v/v) acetonitrile containing 0.05% trifluoroacetic acid at a flow rate of 0.5 ml/min at 40 °C. The amount of product was estimated by measuring its fluorescence intensity at 562 nm, with excitation at 342 nm. Protease activity (pmol/h/µl) in the vitreous fluid was estimated from the standard curve of synthetic Dansyl-D-Ala-Gly fluorescence intensity. Neprilysin-like activity (thiorphan-sensitive peptidase activity) in each sample was evaluated by subtraction of thiorphan-insensitive enzymatic activity (i.e., that recorded in the presence of thiorphan) from total activity.

Measurement of amyloid β: Specimens were collected in sterile tubes and stored at -80 °C until the analysis was conducted. Sensitive, specific enzyme-linked immunosorbent assays (ELISA) specific for Aβ42 were used for the analysis (INNOTEST β-amyloid, INNOTEST, Gent, Belgium). Six out of eight vitreous samples in the proliferative DR group were used to measure Aβ42, because the volume of two samples was insufficient for measurement purposes. All samples were run in duplicate. The experiment was carried out in a masked fashion (by SY).

Statistical analysis: Data are expressed as means±SEM and were analyzed using a one-way ANOVA followed by a Student t-test.

RESULTS

Neprilysin-like activity in vitreous of patients with proliferative diabetic retinopathy: The clinical characteristics of the patients providing vitreous fluid data are given in Table 1. Figure 1 shows the neprilysin-like activity levels in the vitreous of patients with macular hole (Control) or proliferative DR (Patient), with representative chromatograms for the measurement of neprilysin activity being shown in Figure 1A. The values for peptidase activity, obtained using Dansyl-D-Ala-Gly-p-nitro-Phe-Gly, in the Control group (n=8) were 0.92±0.03 and 0.77±0.04 pmol/h/µl (mean±SEM) in the absence and presence of thiorphan, respectively. In the Patient (n=10) group, the corresponding values were 1.45±0.14 and 0.75±0.03 pmol/h/µl in the absence and presence of thiorphan, respectively. There was a significant difference between the Control and Patient groups in total activity (without thiorphan), whereas the thiorphan-insensitive activities of the two groups were almost the same (Figure 1B). The values obtained for neprilysin-like peptidase activity in Control (n=8) and Patient (n=10) groups were 0.15±0.03 and 0.70±0.12 pmol/h/µl, respectively, the mean value for patients with proliferative DR being approximately 4.7 times that of the controls (Figure 1C).

Concentration of amyloid β in vitreous of patients with proliferative diabetic retinopathy: Figure 2 shows the concentrations of Aβ in the vitreous of patients with macular hole (Control) or proliferative DR (Patient). In Control (n=6) and Patient (n=10) groups, these values were 60.9±7.34 and 8.94±3.30 pg/ml, respectively, the latter value being significantly smaller than the former.

Relationship between neprilysin-like activity and amyloid β concentration in vitreous: Figure 3 shows that neprilysin-like peptidase activity was elevated and, in contrast, the Aβ concentration was reduced in the vitreous of proliferative DR patients (versus the controls). There was a significant inverse correlation (y=-0.0089x + 0.7472, p=0.0072, R²=0.4134) between neprilysin-like peptidase activity and Aβ concentration among all subjects.

DISCUSSION

In this study, we observed a significant increase in neprilysin-like peptidase activity and a significant reduction in Aβ concentration in the vitreous fluid of patients with proliferative DR (by comparison with macular hole patients), and a significant inverse correlation was revealed between these two parameters in vitreous fluid.
Recent reports [19,24-27] suggest that Aβ increases in various areas of the eye during the progression of eye diseases, and therefore that the increase in Aβ might contribute to the eye disease. Interestingly, Aβ deposition has been reported to be specific to drusen in the eyes of patients with age-related macular degeneration (AMD) [24-26], suggesting that Aβ might be associated with the more advanced stages of AMD [26]. AMD is a retinal degenerative disease that leads to a loss of central vision, and it affects 5-10% of the population over 60 years of age. A number of studies have confirmed that the presence of drusen-identified as gray-yellow deposits that collect in or around the macula of the retina-represents a significant risk factor for the development of visual loss from AMD [28-30]. Goldstein et al. [27] noted that Aβ is present in the cytosol of lens fiber cells in patients with AD and that the presence of Aβ in the lens might promote regionally specific lens protein aggregation, extracerebral amyloid formation and supranuclear cataracts. Recently, an association between diabetes mellitus and an increased risk of developing AD has been suggested (i.e., diabetes mellitus may be associated with the development of AD and with the decline in cognitive function in older persons) [31]. We reported recently that the concentrations of Aβ42 and tau showed a decrease and an increase, respectively (versus macular hole controls), in the vitreous fluid from patients with glaucoma and DR [19]. However, there have been no previous reports regarding changes in neprilysin in eye diseases.

An increase in TUNEL-positive cells in retinal neurons has been observed at an early stage in streptozotocin-diabetic rats and in human diabetes, and neurodegeneration may be an important component of DR [32]. Given our finding that a decrease in the concentration of Aβ42 in the vitreous fluid obtained from patients with DR is observed regardless of the manner of the disease progression (proliferative or edematous), it is possible that factors associated with the early stages of diabetes development may affect Aβ metabolism in the retina.

**Figure 1. Neprilysin-like peptidase activity in vitreous samples.**

Neprilysin-like peptidase activity was measured in vitreous samples obtained from patients with macular hole (Control) or proliferative diabetic retinopathy (Patient). A: Representative chromatograms for measurement of neprilysin activity. Arrows indicate the product, Dansyl-D-Ala-Gly. B: Peptidase activity in the absence (open column) or presence (solid column) of thiorphan was measured by means of an HPLC-fluorometric system, as described under Materials and Methods. C: Neprilysin-like peptidase activity was calculated by subtraction of thiorphan-insensitive peptidase activity from total activity. Values are mean±SEM, n=8 (Control) and 10 (Patient). Asterisk (*) represents p<0.001 (Student t-test).
possibly leading to neurodegeneration in DR. Further studies will be needed for a full elucidation of the alterations in Aβ in DR.

Recently, we established a novel, sensitive protocol for the measurement of extracellular cell-surface neprilysin activity in living cells (using an HPLC apparatus) [23]. Using this system, we investigated the effect of hypoxia on neprilysin activity in human neuroblastoma SH-SY5Y cells, and we demonstrated that hypoxia (under 5% O₂ for 24 h)-an event closely associated with DR and neurodegenerative diseases-significantly attenuated neprilysin activity without any alteration in neprilysin gene expression [23]. Neprilysin is reported to be secreted from LLC-PK1 cells (originate from pig proximal tubule of the nephron) [33], human prostatic secretory cells [34] and invasive human melanoma cell lines [35]. In case of prostatic tissue, neprilysin is released from the cells in an apocrine fashion [34]. Recently, it was shown that cerebrospinal fluid (CSF) neprilysin elevates with the progression of Alzheimer’s disease, and suggested that presynaptically located neprilysin could be released into CSF as a consequence of synaptic disruption [36]. Therefore, one possibility is that under a hypoxic condition, neprilysin is released from membrane, with the result that soluble neprilysin is increased at the extracellular level.

A limitation of the present study is the small sample sizes. Nevertheless, we observed an apparent relationship between increased neprilysin-like peptidase activity and decreased Aβ concentration in patients with proliferative DR. On the basis of the present data, we can speculate about the underlying mechanism as follows. One of many possible reasons for this observation is degradation of Aβ by neprilysin. Namely, retinal damage is induced by DR, and consequently the neprilysin bound to the membrane of the retinal cells is cleaved off, thereby increasing the concentration of soluble neprilysin within the vitreous body. If membrane-anchored neprilysin serves to offer a locus for the degradation of newly generated Aβ within the synapse, any neprilysin separated from the cell membrane will not be able to perform its role of scavenging synaptic Aβ molecules. It is possible that a decrease in cell-surface neprilysin causes an enhancement of Aβ aggregation within the synapse and a degeneration of ganglion cells within the retina. This hypothesis is consistent with the observation of a decrease in Aβ42 in the vitreous of patients with proliferative DR since some might form aggregates on the membrane of retinal cells, while others might be degraded by soluble neprilysin within the vitreous.

There are other possible mechanisms, as follows: In DR patients, Aβ accumulates in the retina for some reason and consequently Aβ and neprilysin levels in the vitreous decrease and increase, respectively. Aβ is reportedly detected in retinal ganglion cells (by immunohistochemistry) significantly more often in ocular hypertensive retinas than in control retinas [37]. Further work will be necessary to determine whether the decrease in the Aβ level really is related to the increase in neprilysin activity.

In conclusion, neprilysin activity and Aβ concentrations displayed converse changes in patients with proliferative DR, there being a significant inverse correlation between the two parameters.

Figure 2. Concentrations of β-amyloid (1-42) (Aβ42) in vitreous samples. Aβ42 was measured in vitreous samples obtained from patients with macular hole (Control) or proliferative diabetic retinopathy (Patient) by use of sensitive, specific enzyme-linked immunosorbent assays (ELISA), as described under Methods. Values are mean±SEM, n=6 (Control) and 10 (Patient). Sharp (#) indicates p<0.0001 for comparison to control (Student t-test).

Figure 3. Relationship between neprilysin-like peptidase activity and amyloid β concentration in vitreous. Neprilysin-like peptidase activity was elevated and, in contrast, Aβ was reduced in the vitreous of proliferative DR patients (versus Control). There was a significant inverse correlation (y=-0.0089x + 0.7472, p=0.0072, R²=0.4134) between neprilysin-like peptidase activity and Aβ among all subjects.
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