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Original Article

Insensitivity of PI3K/Akt/GSK3 signaling in peripheral blood mononuclear cells of age-related macular degeneration patients

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Abstract

Our recent studies with cultured retinal pigment epithelium cells suggested that overexpression of interleukin 17 receptor C (IL-17RC), a phenomenon observed in peripheral blood and chorioretinal tissues with age-related macular degeneration (AMD), was associated with altered activation of phosphatidylinositide 3-kinase (PI3K), Akt, and glycogen synthase kinase 3 (GSK3). We wondered whether or not altered PI3K, Akt, and GSK3 activities could be detected in peripheral blood mononuclear cells (PBMC) obtained from AMD patients. In the patients' PBMC, absent or reduced serine-phosphorylation of GSK3α or GSK3β was observed, which was accompanied with increased phosphorylation of GSK3 substrates (e.g. CCAAT enhancer binding protein α, insulin receptor substrate 1, and TAU), indicative of enhanced GSK3 activation. In addition, decreased protein mass of PI3K85α and tyrosine-phosphorylation of PI3K50α was present in PBMC of the AMD patients, suggesting impaired PI3K activation. Moreover, abnormally lowered molecular weight forms of Akt and GSK3 were detected in PBMC of the AMD patients. These data demonstrate that despite the presence of high levels of IL-17RC, Wnt-3a and vascular endothelial growth factor, the PI3K/Akt/GSK3 signaling pathway is insensitive to these stimuli in PBMC of the AMD patients. Thus, measurement of PI3K/Akt/GSK3 expression and activity in PBMC may serve as a surrogate biomarker for AMD.

Keywords: phosphatidylinositide 3-kinase (PI3K), protein kinase B (PKB or Akt), glycogen synthase kinase 3 (GSK3), age-related macular degeneration (AMD), peripheral blood mononuclear cells (PBMC)

Introduction

Age-related macular degeneration (AMD) is a chronic disorder characterized by gradual loss of central vision in the elderly[1]. Clinically, early AMD is characterized by histopathologic features of drusen, which are extracellular lipofuscinoid deposits consisting of lipids and proteins (notably apolipoproteins and complements) present between retinal pigment epithelium (RPE) and choriocapillaris of the choroid. Often, early AMD is also associated with pigmentary changes but is largely asymptomatic. Advanced forms of AMD, on the other hand, are often symptomatic and characterized by neovascularization of endothelial cells from choriocapillaris of the choroid migrating toward and even cross the RPE into the retina, a symptom clinically termed "wet" AMD (accounting for 10%-15% of total AMD)[2]. The advanced or late form of AMD, devoid of choroidal neovascularization, is termed "dry" AMD (accounting for 85%-90% of total

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AMD)[2], also known as geographic atrophy, and is characterized by atrophy of RPE cells and choriocapillaris.

Because of the presence of autoantibody, AMD has been considered as an autoimmune disease[3], attributable to frequent auto-activation of complement, a vital component of the innate immunity and an effector mechanism of the innate immune system[4]. Complement can be activated either via the classic pathway, requiring antibodies to recognize foreign/abnormal antigens, or else by foreign/abnormal antigens via the alternative pathway[4]. Complement consists of many regulators/components so that the system is highly regulated and any mutation in those proteins can increase risks of the system disorders. For example, a gene mutation of complement regular factor H (Y402H) has been linked to increased risk of AMD[5].

AMD is also associated with interleukin-17 receptor (IL-17RC); high-level expression of IL-17RC has been observed in the macular tissues as well as in $CD14^+$ monocytes of the AMD patients[6]. Although inconclusive, increased IL-17RC expression may be attributable to DNA hypomethylation at the *IL-17RC* promoter region[6,7]. We have recently shown that overexpression of IL-17RC in cultured RPE cells resulted in augmented expression of not only Wnt-3a and Wnt-10b but also the Wnt-target gene vascular epidermal growth factor (VEGF)[8]. Activation of the canonical Wnt pathway is known to effectively induce retinal inflammation and play a pathogenic role in AMD development[9]. Overexpression of IL-17RC can enhance protein–protein interactions between β-catenin and VEGF, as well as interactions between glycogen synthase kinase 3β (GSK3β) and VEGF[8]. The β-catenin/VEGF and GSK3β/VEGF aggregates were co-localized with C3, the first reactive component of the alternative complement pathway[4]. It was thus hypothesized that the β-catenin/VEGF and GSK3β/VEGF aggregates may be recognized by the alternative complement pathway as foreign/abnormal antigens[8].

In addition to increased β-catenin/VEGF and GSK3β/VEGF interactions with C3, overexpression of IL-17RC in RPE or THP-1 (cultured monocytes) cells also resulted in the loss of responsiveness of Akt and/or phosphatidylinositide 3-kinase (PI3K) to stimuli such as VEGF[8]. The insensitivity of PI3K/Akt was associated with increased GSK3 activity, as well as with decreased cell growth and cell survival[8], consistent with the observation that high GSK3 activity promotes apoptosis[10–12]. Thus, the uncontrolled GSK3 activation under high IL-17RC and VEGF conditions has been postulated to confer an etiology of AMD that is unrelated to complement system activation[8].

Peripheral blood mononuclear cells (PBMC) consist of any peripheral blood cells having a nucleus, including lymphocytes (e.g. T cells, B cells and natural killer cells) and monocytes. Because IL-17RC levels are increased in monocytes of AMD patients[6] and because forced expression of IL-17RC increases GSK3 activity in transfected RPE cells[8], we wondered if high GSK3 activity and its related effects can also be detected in PBMC of AMD patients.

Materials and methods

Human blood samples

Blood samples of two AMD patients were collected at the laboratory of Dr. Robert Nussenblatt, Immunology, National Eye Institute, the US National Institutes of Health (NIH). Two normal blood samples were obtained from NIH blood bank under Dr. Nussenblatt's name. Those people, donating their blood as normal samples, did not have any common chronic diseases including AMD. Informed written consent for participation was obtained from the donors to NIH as described previously[6], and the sample handling and subsequent experimental procedures were strictly following protocols stipulated by NIH review boards. The ages of the two AMD patients were matched with normal controls.

Preparation of PBMC

PBMC were isolated from 5 to 10 mL blood samples using Ficoll and a series of centrifugation according to a previously described protocol[6].

Immunoblotting and semi-quantification

Cell lysate preparation, immunoblotting procedures, antibodies and their sources have been described[8]. The antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) were: α-Akt (#sc-8312), α-c-Myc (#sc-788), α-cyclin D1 (#sc-718), α-GSK3 (#sc-56913), α-GSK3α (#sc-7879), α-GSK3β (#sc-9166), α-IL-17RC (#sc-99936), α-insulin receptor substrate 1 (IRS1) (#sc-559), α-pAkt (#sc-135650), α-pGSK3β (#sc-11757-R), α-PI3K (#sc-423), α-pPI3K (#sc-12929-R), α-TAU (#sc-1995), α-VEGF (#sc-152), and α-Wnt-3a (#sc-136163). The α-CCAAT enhancer binding protein α (c/EBPα) (#2295), α-pc/EBPα (#2844), and α-pIRS1 (#2580) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA), and the α-GAPDH antibody (#2-RGM2) was obtained from Advanced ImmunoChemical (Long Beach, CA, USA). After SDS-PAGE and transfer, the blots were blocked by 5% bovine serum albumin (Sigma Aldrich, St. Louis, MO, USA) in the described buffer[8] for one hour. Then, the

blots were incubated with different primary antibodies (rabbit antibodies: 1:500; mouse antibodies: 1:200) overnight. The blots were washed by the buffer for three times and then incubated with corresponding secondary antibodies conjugated with Horseradish peroxidase (1:1,000 for rabbit antibodies and 1:200 for mouse antibodies) for one hour. The blots were washed by the buffer twice and washed by the buffer without Tween-20 once. The enhanced chemiluminescence technique was applied to visualize the immuno-reactive proteins. The immunoblots were exposed for different period of times to ensure linearity of the intensity of protein bands, prior to semi-quantification by scanning densitometry using NIH Image J.

Statistical analysis

The scanned results of intensity protein bands from PBMC of normal or AMD donors by two separate experiments were compared. P values were calculated using Wilcoxon-Mann–Whitney Test (WMWT) (https://ccb-compute2.cs.uni-saarland.de/wtest/ id = www/www-ccb/html/wtest).

Results

Increased levels of IL-17RC, Wnt-3a, and VEGF in PBMC of AMD patients

Elevated frequency of IL-17RC$^+$CD14$^+$ monocytes has been observed previously in the peripheral blood of AMD patients, and the increased IL-17RC expression (in both peripheral blood and chroioretinal tissues with AMD lesions) probably was associated with hypomethylation of the *IL17RC* promoter[6]. The present study confirmed, using immunoblot analysis, that the level of IL-17RC was increased (by 2.3-fold) in PBMC of two AMD patients examined. Increased IL-17RC expression was shown to induce Wnt signaling and Wnt-3a and Wnt-10b expression in cultured retinal pigment epithelium[8]. Indeed, the level of Wnt-3a in PBMC of the two AMD patients was increased (by 4.1-fold) as compared to that in non-AMD controls (***Fig. 1***). Moreover, like that in retinal pigment epithelium with AMD lesions[1], increased level of VEGF (by 12.7-fold) was also observed in PBMC of AMD patients (***Fig. 1***). These results thus provide the first evidence that elevated expression of IL-17RC, Wnt-3a, and VEGF, factors strongly associated with AMD can be detected by immunoblotting in PBMC of AMD patients.

Insensitivity of PI3K/Akt/GSK3 signaling in PBMC of AMD patients

We have shown previously that in transfected RPE cells overexpressing IL-17RC, tyrosine phosphorylation (Y208) of PI3K50α was abrogated even though the level of VEGF, a PI3K stimulus, was persistently high[8]. Concomitantly, the decreased pYPI3K50α was associated with reduced threonine phosphorylation

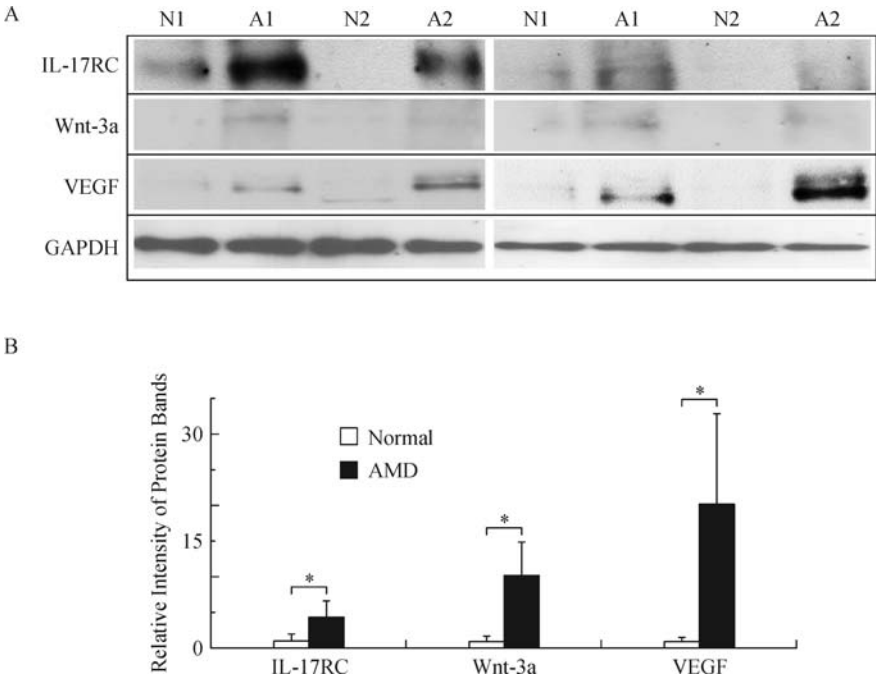

Fig. 1 **Immunoblot analysis of IL-17RC, Wnt-3a, and VEGF.** A: Immunoblots of IL-17RC, Wnt-3a and VEGF from PBMC of two normal (N) and two AMD (A) donors. GAPDH is the loading control. B: Semi-quantification of intensity of the respective IL-17RC, Wnt-3a and VEGF bands by scanning densitometry. The normal control value is set as one. Data are averaged from 4 values from each condition for each of the proteins and presented as mean±SD. * $P < 0.05$.

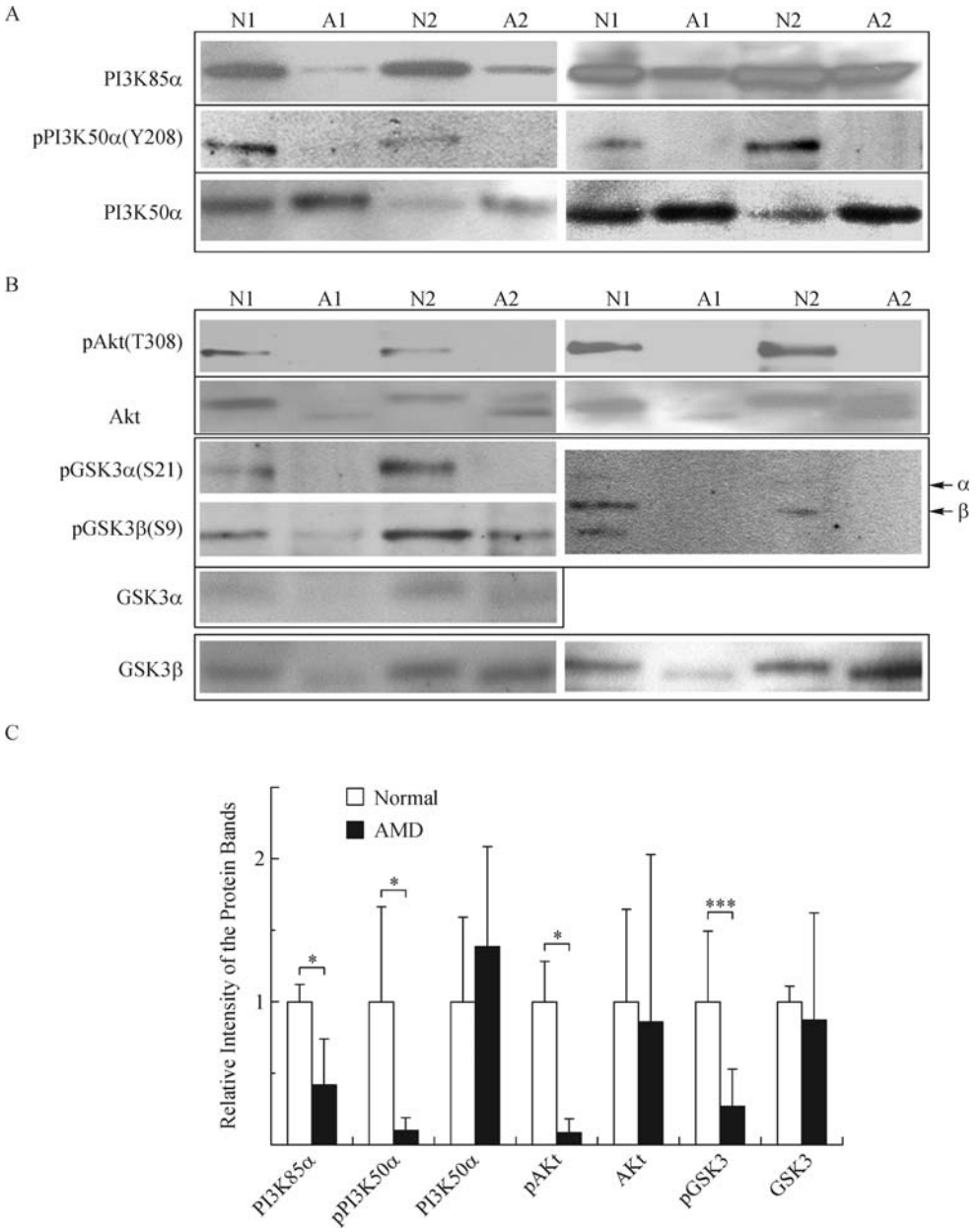

Fig. 2 **Immunoblots analysis of protein mass and phosphorylation status of PI3K, Akt, and GSK3.** The PBMC of normal control and AMD patients were obtained as in ***Fig. 1***. A: Immunoblots of PI3Ks as indicated including two separate experiments. B: Immunoblots of Akt and GSK3 as indicated including two separate experiments except GSK3α. The arrows at the right of the pGSK3 blot indicate the bands of pGSK3. The arrows at the left of the bolts indicate the lower molecular bands of Akt, GSK3α and GSK3β, respectively. C: Semi-quantification of the intensity of PI3K85α, pPI3K50α, PI3K50α, pAkt, Akt, pGSK3 and GSK3 bands. Data are averaged from 4 values (PI3K85α, pPI3K50α, PI3K50α, pAkt or Akt), 8 values (pGSK3) or 6 values (GSK3) from each condition and presented by mean±SD. * $P<0.05$, *** $P<0.001$.

(T308) of Akt, and lowered levels of serine-phosphorylated GSK3[8], indicative of GSK3 activation. To determine whether or not the insensitivity of PI3K/Akt/GSK3 signaling to VEGF stimulus was also detectable in PBMC of the AMD patients, we performed immunoblot analysis of the phosphorylation status of the respective PI3K α subunits (*i.e.* the regulatory subunit of PI3K), Akt, and GSK3.

There are three alternative splicing isoforms of PI3K regulator α subunits, namely PI3K85α, PI3K55α, and PI3K50α derived from the same gene (NM_001242466.1, human)[13]. The PI3K55α was not detectable in PBMC since there was no band between the bands of 50-kDa and 85-kDa. The level of PI3K85α in AMD PBMC was decreased by 60% as compared to that in normal controls (***Fig. 2A***). More remarkably, the level of tyrosine-phosphorylated pPI3K50α(Y208), representing the activated form of PI3K, was reduced

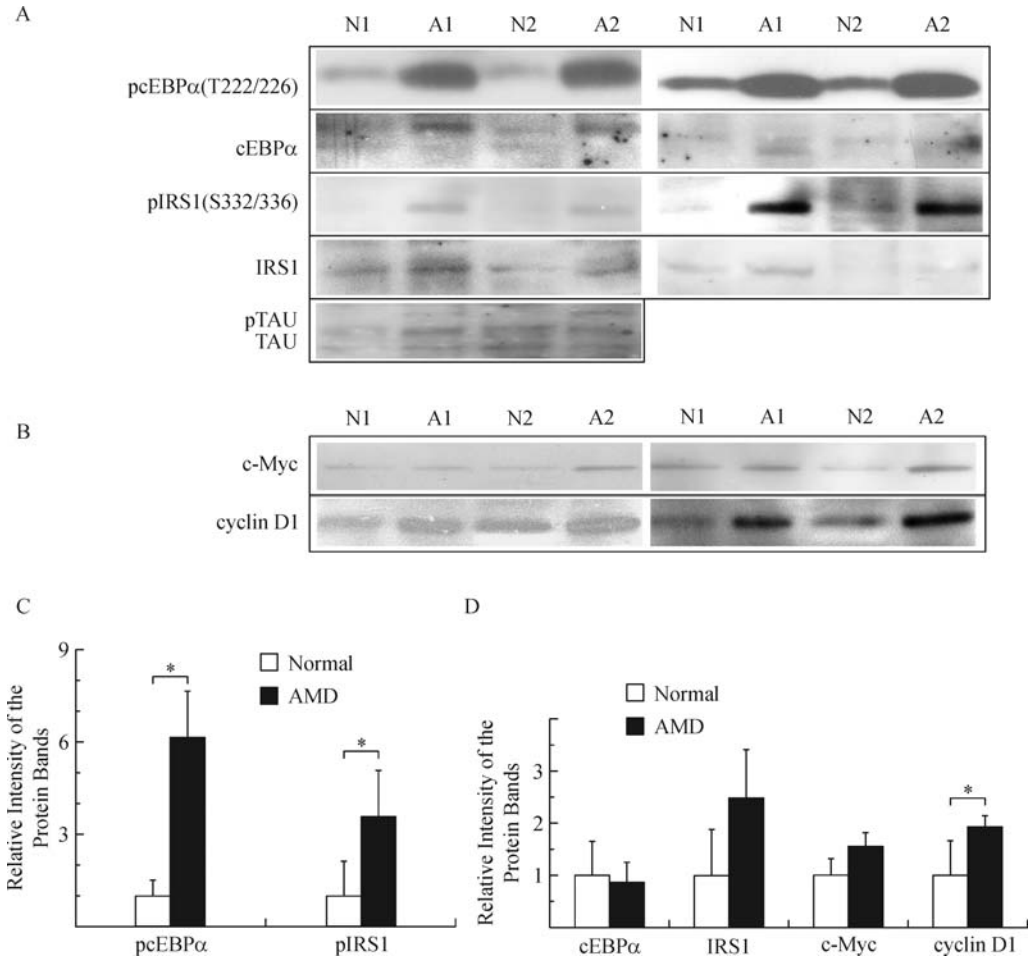

Fig. 3 **Immunoblots analysis of protein mass and phosphorylation status of GSK3 substrates.** The PBMC of normal control and AMD patients were obtained as in ***Fig. 1***. A: Immunoblots of the GSK3 substrates as indicated, including two separate experiments except for TAU where only one experiment was performed. The top arrow indicates the phosphorylated band of TAU, which was presented in AMD samples and not in PBMC of normal controls. The bottom arrow indicates non-phosphorylated TAU. B: Immunoblots of c-Myc and cyclin D1 as indicated, including two separate experiments. C: Semi-quantification of the intensity of pc/EBPα, and pIRS1 bands. Data are averaged from 4 values from each condition for each of the proteins and presented by mean±SD. * $P < 0.05$ (AMD versus normal control). D. Semi-quantification of the intensity of c/EBPα, IRS1, c-Myc, and cyclin D1 bands. Data are averaged of 4 values from each condition for each of the proteins and presented by mean±SD. * $P < 0.05$.

to only10% of that in normal controls (***Fig. 2A*** and ***C***).

Two Akt bands were present in PBMC of the AMD patients, neither of which was phosphorylated. Thus, the level of threonine-phosphorylated Akt, pAkt(T308), was less than 10% of that in normal controls (***Fig. 2B*** and ***C***), suggesting that Akt was minimally activated in PBMC of the AMD patients. The nature of the low molecular weight form of Akt (denoted by an arrow in ***Fig. 2B***) present in PBMC of the AMD patients is unclear; it might be a degradative product of Akt due to structural damage.

Both GSK3α and GSK3β were detected in PBMC of the AMD patients (***Fig. 2B***). However, the serine-phosphorylated pGSK3α (S21) was undetectable, and the apparent molecular weight of GSK3α (denoted by an arrow) was lower in PBMC of AMD patients than that in normal controls (***Fig. 2B***). The level of serine-phosphorylated pGSK3β (S9) was reduced by over 50% in PBMC of the AMD patients, and the apparent molecular weight of GSK3β (denoted by an arrow) was also lower in these cells as compared with that in normal controls (***Fig. 2B***). The absence of pGSK3α (S21) and reduced levels of pGSK3β (S9) are indicative of enhanced GSK3 activities in PBMC of the AMD patients. If the levels of pGSK3α and pGSK3β were combined, the serine-phosphorylation status of GSK3 in PBMC of AMD patients was reduced by over 70% as compared to normal controls (***Fig. 2C***). However, the levels of protein mass of PI3K50α, Akt, or GSK3 were insignificantly changed between normal and AMD PBMCs (***Fig. 2C***). These results together suggest that despite the presence of high levels of IL17RC, Wnt-3a

or VEGF, the PI3K/Akt signaling is insensitive to these stimuli, which leads to uncontrolled high GSK3 activity in PBMC of the AMD patients.

Increased phosphorylation levels of GSK3 substrates in PBMC of AMD patients

We next determined whether or not the increased GSK3 activity in PBMC of AMD patients was associated with high-level phosphorylation of GSK3 substrates. To this end, we examined c/EBPα, IRS1, and TAU, As shown in ***Fig. 3A***, the levels of pc/EBPα (T222/226), pIRS1(S332/336) and pTAU (denoted by an arrow) were all increased in PBMC of the AMD patients as compared to that in normal controls (***Fig. 3A*** and ***C***). The protein mass of c/EBPα or IRS1 was insignificantly altered between normal and AMD samples (***Fig. 3D***).

Finally, we determined whether or not the increased Wnt-3a in PBMC of the AMD patients was associated with increased expression of c-Myc or cyclin D1, because it is known that Wnt-signaling increases c-Myc and cyclin D1 gene expression[14]. Indeed, the levels of c-Myc and cyclin D1 were augmented over 50% and 90%, respectively, in PBMC of the AMD patients (***Fig. 3B*** and ***D***). This result is consistent with the previous observation that high GSK3 activity had little impact on c-Myc and cyclin D1 expression in RPE or THP-1 cells overexpressing IL-17RC because of high levels of Wnt-3a and Wnt-10b[8].

Together, these data show that the attenuated PI3K/Akt response to the stimuli and the concomitant release of GSK3 activity are apparent in PBMC of the AMD patients, suggesting that the overstimulation-induced PI3K/Akt/GSK3 insensitivity and GSK3 activation are a systematic manifestation in AMD.

Discussion

On the basis of recent experimental observation that elevated IL-17RC expression was present in peripheral blood samples of AMD patients[6] and that overexpression of IL-17RC in transfected RPE or monocytes was associated with uncontrolled GSK3 activation[8], we postulated that the etiology of AMD might be related to overstimulation-induced-insensitivity of the PI3K/Akt/GSK3 pathway and uncontrolled (or released) GSK3 activity[15]. To test this hypothesis, we examined the phosphorylation status of PI3K, Akt, and GSK3, as well as that of GSK3 substrates in PBMC of AMD patients. Despite the presence of low or comparable expression of GSK3 in PBMC of AMD1 or AMD2 patients (***Fig. 1A***), the activity of GSK3 was much higher than that in PBMC of normal controls, as demonstrated by the high phosphorylation status of GSK3 substrates c/EBPα, IRS1, and TAU (***Fig. 3***). These data, albeit preliminary because only two AMD samples were obtained, demonstrate that the uncontrolled GSK3 activation, a hallmark of overstimulation-induced-insensitivity of PI3K/Akt/GSK3, can be detected in PBMC of the AMD patients.

The etiology/onset of AMD is complex and most likely multifactorial. Many genetic risk factors[16] and biomarkers[17] for AMD have been suggested; the majority of them are associated with the complement system proteins. Nevertheless, because the most relevant risk factor for AMD is aging, and because development of pathogenic AMD takes many years to be evident, identification of a reliable biomarker for AMD in the sub-health populations is of significance in preventative medicine.

We therefore propose that the GSK3 activity assay in PBMC can be used as a surrogate biomarker for AMD. We propose to use GSK3 activity as an AMD biomarker for the following two reasons.

First, GSK3 activation is present in many chronic/age-related metabolic abnormalities, including AMD[18]. Association of AMD with other chronic/age-related metabolic abnormalities, such as type 2 diabetes (T2D), and Alzheimer disease, has been reported previously. For instance, the Reykjavik Study has shown that late AMD being a predictor of mortality at mid-octogenarian years[19]. An association of diabetes with neovascular AMD, but not with geographic atrophy, has been observed in a cross-sectional population-based study with patients of >65 years old (EUREYE study)[20]. A 10-year cardiovascular mortality and risk factor study suggested AMD be an independent risk factor of cardiovascular mortalities in T2D patients[21], even though a systematic meta-analysis does not reveal a significant relationship between early AMD and incident stroke[21]. Importantly, high GSK3 activity has been observed in many of these chronic/age-related diseases, including Alzheimer's disease, T2D[22], Parkinson disease[23], and even cancer[24,25]. The GSK3-catalyzed serine-phosphorylation of IRS1 plays a crucial role in the development of T2D[26], and GSK3-catalyzed hyper-phosphorylation of TAU has been implicated in the development of Alzheimer's disease[27]. In the present study, we observed increased phosphorylation of IRS1 and TAU, as well as increased phosphorylation of c/EBPα in PBMC of the AMD patients (***Fig. 3***). This result, although preliminary, may suggest that uncontrolled GSK3 activation is a systematic manifestation of many chronic/age-related disorders with a common mechan-

istic underpinning, *i.e.* overstimulation-induced-insensitivity of PI3K/Akt/GSK3 pathway[15].

Second, measurement of uncontrolled GSK3 activation can serve as a good diagnostic means for patients under sub-health or chronic conditions, such as early stages of AMD and/or other chronic diseases. Currently, most of the genetic risk scores or biomarkers, except aging, have not been able to (i) reliably predict the development of AMD or (ii) the effectiveness of drugs or therapies[17]. This challenge in clinical practice is most likely attributable to the lack of knowledge on the causal relationship between AMD and inflammatory responses, as well as the lack of knowledge of metabolic baseline of selected "normal" or "control" groups. The recent controversy concerning the use of hypo-methylation status of the *IL17RC* promoter as an indicator for AMD may be attributable to such a lack of a suitable "control" population[6-7]. Our recent elucidation of an interrelationship between IL-17RC-, Wnt-, VEGF-stimulation and insensitivity of PI3K/Akt/GSK3 signaling[8] has provided a mechanistic explanation of the etiology of AMD at molecular and cellular levels. We have observed changes in GSK3 activity as early as two days upon overstimulation[8], which may represent a pre or early stage of diseases. Therefore, the assay GSK3 activity can be applied to distinguish patients with chronic/sub-health conditions from the healthy subjects, and also to help with evaluation of safety and effectiveness of drugs/therapies.

It is noteworthy that the insensitivity of PI3K/Akt/GSK3 in PBMC of the AMD patients was much more severe than that in cultured cells with overstimulation[8], because the level of PI3K85α was dramatically diminished (***Fig. 2A*** and ***C***) and the presence of low molecular weight forms of Akt and GSK3 in the cells (***Fig. 2B***). These phenomena were not observed in cell culture studies[8], nor were they present in animal studies reported previously[15]. Mechanisms by which persistent internal expression of the stimuli (***Fig. 1***) can exert such a detrimental effect on cellular expression of PI3K, Akt and GSK3 merit further investigation.

In summary, measurement of mass and phosphorylation status of GSK3 and GSK3 substrates in PBMC may supplement other clinical biochemistry tests in the surveillance of AMD risks among peoples who carry genetic risks of the disease.

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