β3-integrin Leu33Pro gain of function variant does not modulate inflammatory activity in human derived macrophages in diabetes

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Abstract

Objective: We aimed to investigate the association between the Leu33Pro (rs5918) polymorphism in β3-integrin with diabetic complications and inflammatory function of macrophages depending on the genotype in subjects with diabetes mellitus.

Material and methods: We determined the Leu33Pro polymorphism in 186 diabetic subjects and collected laboratory data. Monocytes from 24 patients were collected for macrophage differentiation to determine the inflammatory activity by treating with different stimulants.

Results: We could demonstrate that human derived differentiated macrophages expressed β3-integrin. Their secretory capacity upon inflammatory stimulation did not reveal any differences depending on the Leu33Pro variant. We found trends for an association of the polymorphism with the presence of diabetic nephropathy (p = 0.071), as well as with creatinine [1.32 mg/dL (1) vs. 0.98 mg/dL (0)] (p = 0.029 in recessive model) and glomerular filtration rate [75.6 ml/min ± 22 vs. 62.3 ml/min ± 25] (p = 0.076 in recessive model) as quantitative markers of kidney function.

Conclusion: Despite the expression of β3-integrin in human macrophages, the Leu33Pro polymorphism in β3-integrin does not modify the inflammatory response upon stimulation but might play a role in the progression of diabetic nephropathy. Further studies are necessary to substantiate such a hypothesis.

Key words: Leu33Pro polymorphism, rs5918, diabetes, diabetic nephropathy, inflammation, macrophages

Introduction

αvβ3-integrin (aka vitronectin receptor) is a heterodimeric cell surface receptor with varying functions depending on the level and location of its cellular expression. It is involved in signaling both between cells, and between cells and the extracellular matrix [1]. αvβ3-integrin ligation induces conformational changes that lead to the facilitation of signaling events within the cells with consequences such as migration, proliferation, and differentiation [2]. Gene-knockout studies in mice suggested a role for αvβ3-integrin in disease conditions such as inflammation, bone diseases and cancer [3-5]. Consequently, β3-integrin modulation, as a part of the vitronectin receptor, has been tested to reduce osteoporosis, tumor growth and angiogenesis by blocking β3-integrin with antibodies or RGD peptide antagonists, yet the results are inconsistent. In clinical studies a natural modulation of β3-integrin by the Leu33Pro polymorphism (rs5918) has been demonstrated to be associated with diabetes mellitus
and furthermore diabetic vascular diseases [6, 7]. The mechanism of this observation remains unclear so far. However, the Leu33Pro polymorphism results in a constitutively active β3-integrin [8] and β3-integrin-deficient macrophages have been shown to be involved in inflammatory atherosclerosis [3].

The aim of our study was to investigate if there is an association between the Leu33Pro polymorphism with diabetic complications and to functionally characterize the inflammatory activity of human differentiated macrophages in carriers of the polymorphism versus controls.

**Material and Methods**

The study was approved (11 June, 2015) by the ethics committee of Saarland (E242/14) and all participants gave written consent to the study (n=193). They were recruited from collaborating diabetic clinics and diagnosis of diabetes was made according to American Diabetes Association criteria. 186 subjects (62% men, 38% females; mean age 64 years) were included into the study. Patient characteristics and biochemical parameters were determined from standardized clinical charts. The patients were genotyped for the Leu33Pro polymorphism using a Taqman assay modified from a previously published protocol [9]. Blood was drawn from the study participants (n=24) for harvesting blood derived monocytes in Leucocept tubes (Greiner, Kremsmuenster, Austria) through Ficoll-Paque Premium density gradient centrifugation. CD14-expressing cells were selected using CD14+ micro-beads for incubation and magnetic activated cell sorting (MACS, Miltenyi biotech, Bergisch-Gladbach, Germany). The CD14+ cells were further differentiated into macrophages by incubation with X-VIVO™ 10 medium containing 100 ng/ml M-CSF for 8 days. The species and vitality of the cultured cells were confirmed by immunofluorescence. Macrophages were plated in equal numbers in 12 well plates and, after serum starvation, stimulated either with 100 ng/ml LPS, 200 nM PMA or 50 ng/ml interferon γ. MPO- and multiplex ELISAs were performed on cell supernatants according to manufacturer’s instructions and normalized to cell protein concentration. Statistical methods comprised parametric and non-parametric correlation analyses, Chi-square test, ANOVA or non-parametric tests where appropriate. Statistical analyses were performed using SPSS, release 19 (IBM SPSS Statistics, Chicago).

**Results**

The allele frequencies were 0.84 for the wildtype and 0.16 for the Leu33Pro polymorphism. The allele frequencies were in accordance to the Hardy-Weinberg-equilibrium (Chi-square p=0.59). The characteristics of the study population according to the genotype are displayed in Table 1. The cultivated monocytes from the diabetic patients with the different genotypes were differentiated into macrophages. Apart from morphology, we assessed the successful differentiation by verifying for expression of the macrophage specific marker EMR-1 and proper uptake of oxLDL by immunofluorescence to prove the vitality of the cultured cells (Fig. 1A, B, C). We could also show that β3-integrin is expressed in unstimulated differentiated macrophages (Fig. 1D). We next examined the macrophage secretory capacity in response to the stimuli (LPS, PMA, Interferon γ) depending on the appropriate genotype. In this regard, we found no unifying and logical association of a genotype with the magnitude of any of the measured inflammatory markers.

**Table 1.** Clinical characteristics of patients according to the Leu33Pro polymorphism; values as mean ± SD or median with interquartile difference.

| Factor                        | Wild-type (n=131) | Heterozygous (n=49) | Homozygous (n=6) |
|-------------------------------|------------------|--------------------|-----------------|
| Age (years)                   | 65 (10)          | 62 (16)            | 70 (40)         |
| BMI (kg/m²)                   | 29.4 (6)         | 30.0 (7)           | 30 (11)         |
| Cholesterol Total (mg/dL)     | 193.1 ± 41       | 191.6 ± 31         | 181.8 ± 35      |
| HDL (mg/dL)                   | 46.7 (21)        | 46.2 (12)          | 46.8 (32)       |
| LDL (mg/dL)                   | 119.8 ± 35       | 191.6 ± 31         | 181.8 ± 35      |
| Triacylglycerol (mg/dl)       | 175 (114)        | 167 (149)          | 118 (59)        |
| Fasting plasma glucose (mg/dL)| 153 (55)         | 130 (81)           | 157 (172)       |
| HbA1c (%)                     | 7 (2)            | 7.2 (2)            | 7.5 (2)         |
| CRP (mg/dL)                   | 0.95 (5)         | 0.28 (2)           | 0.41 (0)        |
| Creatinine (mg/dL)            | 0.97 (0)         | 1 (0)              | 1.32 (1)        |
| GPT (U/L)                     | 26.5 (18)        | 28 (18)            | 18.5 (9)        |
| GGT (U/L)                     | 35 (31)          | 27 (28)            | 32 (49)         |
| Uric acid (mg/dL)             | 5.9 (2)          | 5.7 (2)            | 8.3 (4)         |
| RR (mmHg)                     | 138 (26) / 80 (18)| 139 (18) / 80 (4)  | 133 (43) / 82 (18)|
| GFR (ml/min/1.73m²)           | 73.4 ± 21        | 81.7 ± 22          | 62.3 ± 25       |
Previous results suggested an association between the Leu33Pro variant in β3-integrin with macrovascular complications in diabetic patients [10]. Here, we did not find a significant difference in the prevalence of diabetic micro- and macrovascular complications associated with the Leu33Pro polymorphism. We found that the relative frequency of diabetic nephropathy was 50% within the diabetic patients who were homozygous for the Leu33Pro polymorphism, 21% in diabetic patients homozygous for the wildtype, and 12% in the heterozygous diabetic patients. We also found indications of significant differences in GFR depending on the genotype in the overall ANOVA (p=0.034) accompanied by a corresponding trend (p=0.112) towards higher creatinine levels in the homozygous versus the wildtype group [1.32 mg/dL (0) vs. 0.97 mg/dL (0)].

Due to the overall low number of homozygous subjects in our study, we employed a recessive model to leave out one degree of freedom weight as compared to setting out 3 genotypes as separate categories. In this recessive model which requires homozygosity to show an effect we found a significant increase (p=0.029) of the creatinine levels in homozygosity (Fig. 1) as well as a trend towards reduced GFR rate (p=0.076) in the homozygous subjects as compared to the control group of heterozygous and wildtype patients.

**Conclusions**

The exact pathophysiology of the diabetes complications remains incompletely understood. In addition to the toxicity of hyperglycemia, inflammatory processes also likely make an important contribution to the pathophysiology. Especially
macrovacular complications appear to be a result of inflammatory vascular diseases and the β3-integrin has been implicated in inflammatory diseases and diabetes [3, 6, 11]. These observations prompted us to examine macrophages from diabetic subjects carrying the Leu33Pro allele of β3-integrin. There are previous reports claiming that β3-integrin is not expressed in macrophages at all [12], and yet, we were able to show that β3-integrin is expressed in fully differentiated human macrophages (Fig. 1). To our surprise, we did not find a significantly and/or coherently altered inflammatory capacity do not contribute to the explanation of the observed association. The missing underlying explanation warrants further basic research efforts and epidemiological evidence.

Abbreviations

SNP: single nucleotide polymorphism; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein; SNP: single nucleotide polymorphism; GFR: glomerular filtration rate; LPS: lipopolysaccharide; PMA: phorbol-12-myristate-13-acetate; RGD: tripeptide arginine, glycine, and aspartate.

Author Contributions

PH and ED performed the experiments and collected the data, KR and SS contributed to the data interpretation and helped writing the paper. JGS collected the data, KR and SS contributed to the data interpretation and helped writing the paper. PH and ED contributed to the study protocol and analyzed data. All authors interpreted the findings, edited and approved the final version of the manuscript.

Competing Interests

The authors have declared that no competing interest exists.

References

1. Schneider JG, Schiekofer S, Dugi KA, et al. The proline 12 alanine substitution in the ppargamma2 gene is associated with increased extent of coronary artery disease in men. Exp Clin Endocrinol Diabetes. 2009; 117: 519-521.
2. Hyman RO, et al. Integrins: Bidirectional, allostERIC signaling machines. Cell. 2002; 110: 673-677.
3. Schneider JG, Zhu Y, Semenovitch CF, et al. Macrophage beta integrin suppresses hyperlipidemia-induced inflammation by modulating trnf alpha expression. Arterioscler Thromb Vasc Biol. 2007; 27: 2699-2706.
4. Su X, Eiser AK, Roop K, et al. Antagonizing integrin β3 increases immnosuppression in cancer. Cancer Res. 2016; 76: 3484-3495.
5. Reynolds LE, Wyder L, Hodivala-Dilke KM, et al. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med. 2002; 8: 27-34.
6. Tschoepe D, Menart B, Roesen P, et al. Genetic variation of the platelet-surface integrin gplfilb-IIla (pia1/a2-snp) shows a high association with type 2 diabetes mellitus. Diabetologia. 2003; 46: 984-989.
7. Stratmann B, Xu T, Illig T, et al. Pla1a2 platelet polymorphism predicts mortality in prediabetic subjects of the population based kora s4-cohort. Cardiovascular diabetes. 2014; 13, 90.
8. Jallu V, Poullain P, de Brevont AG, et al. Modeling and molecular dynamics simulations of the v33 variant of the integrin subunit β3: Structural
comparison with the I33 (hpa-1a) and P33 (hpa-1b) variants. Biochimie. 2014; 105: 84-90.

9. Rzezniczek S, Obuchowicz M, Pile A, et al. Decreased sensitivity to paroxetine-induced inhibition of peripheral blood mononuclear cell growth in depressed and antidepressant treatment-resistant patients. Translational psychiatry. 2016; 6: e827.

10. Verdoia M, Secco GG, De Luca G, et al. Platelet hpa-1 a/hpa-1 b polymorphism and the risk of periprocedural myocardial infarction in patients undergoing elective pci. Platelets. 2014; 25: 367-372.

11. Maile LA, Busby WH, Patel A, et al. Blocking ligand occupancy of the avβ3 integrin inhibits the development of nephropathy in diabetic pigs. Endocrinology. 2014; 155: 4665-4675.

12. Zhao H, Ross FP, Teitelbaum SL, et al. Unoccupied alpha(β3-integrin regulates osteoclast apoptosis by transmitting a positive death signal. Mol Endocrinol. 2005; 19: 771-780.

13. Antonov AS, Antonova GN, Verin AD, et al. Alphavbeta3 integrin regulates macrophage inflammatory responses via p3-kinase/akt-dependent nf-kappab activation. J Cell Physiol. 2010; 226(2): 469-76.

14. Lim J, Lai S, Heng CK, et al. Variation of the platelet glycoprotein iiiα pi(a1/a2) allele frequencies in the three ethnic groups of singapore. International journal of cardiology. 2003; 90: 269-273.

15. Jin DK, Fish AJ, Kim Y, et al. Distribution of integrin subunits in human diabetic kidneys. Journal of the American Society of Nephrology. 1996; 7: 2636-2645.

16. Staeck O, Slowinski T, Khadzhynov D, et al. Recurrent primary focal segmental glomerulosclerosis managed with intensified plasma exchange and concomitant monitoring of soluble urokinase-type plasminogen activator receptor-mediated podocyte β3-integrin activation. Transplantation. 2013; 99: 2593-2597.