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RESEARCH ARTICLE

MOLECULAR BIOLOGY

ABERRANT EXPRESSIONS OF HAEMOSTATIC GENES IN GRANULOCYTES OF CHRONIC MYELOPROLIFERATIVE DISORDERS-POLYCYTHAEMIA VERA [PV] AND ESSENTIAL THROMBOCYTHAEMIA [ET]

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ABSTRACT

Objective: A pilot study to test the hypothesis that **dysregulated expression of haemostasis genes in clonal granulocytes of MPD patients contributes to the genesis of haemostatic complications**. In this study of 36 patients with MPD and 18 healthy controls, specific target genes Procoagulant Tissue Factor [TF], prothrombotic cytokines Interlukin1 β [IL1β],Tumor Necrosis Factor α [TNFα],Vascular Endothelial Growth Factor[VEGF] and Heparanase in clonal granulocytes were analyzed by qualitative methods, for disease associated aberrant gene expression, as proof of concept.

Context : Granulocytes especially PMN leukocytes are well known mediators of thrombosis in inflammatory states and leukocytosis, a defining feature of MPD, correlates well with thrombosis, the risk falling with cytoreduction. Further, *in vivo* activation of leukocytes and enhanced platelet-leukocyte interactions have been documented in MPD. Further research is needed to explore the links between leukocytes and the haemostatic complications of MPDs.

Design: This observational study has compared patterns of gene expressions in granulocytes of patients with PV and ET by qualitative RT-PCR.

Patients and Measurements: We studied 14 patients diagnosed with PV, 22 with ET at the Haematology department, and 18 healthy controls . Granulocytes were purified by Ficoll gradient centrifugation, RNA isolated and RT PCR performed using cDNA under optimized conditions. Finally PCR products were separated by Agarose gel electrophoresis and identified by UV transillumination.

Results: TF was expressed by none of the controls but 42% of PV and 27% of ET patients granulocytes revealed TF expression.

Conclusion: Upregulation of TF and other haemostasis related genes in granulocytes genes as suggested by this qualitative method of RTPCR suggests altered gene expression in granulocytes of MPD patients. This could be further explored by quantitative studies.

KEY WORDS

RT PCR expression -TF,Heparanase,TNFα,IL-1β,VEGF and PAR-2 genes-granulocytes of PV and ET - hypercoagulabe state.

INTRODUCTION

Thrombotic and bleeding complications together constitute the major presenting features in MPD patients. They are the major contributors to disease-associated morbidity and the leading cause of death in MPD.

The pathogenesis of haemostatic complications in MPD remains unexplained even today despite years of research. From the literature it can be concluded that the haemostatic complications are not entirely explainable by red cell or platelet abnormalities[1]. Documented significant leukocytosis and its association with thrombosis in MPD patients, along with the observations from functional studies, suggest that leukocytes play a major role in haemostasis. Granulocytes, especially PMN leukocytes, are well known mediators of thrombosis in inflammatory states and other hypercoagulable conditions[2]. Leukocytosis, a defining feature of MPD, correlates well with thrombosis, the risk falling with cytoreduction[3]. Further, *in vivo* activation of leukocytes and enhanced platelet-leukocyte interactions have been documented in MPD[1]. Thus leukocytes appear to be strong candidates involved in the pathogenesis of these complications and many have stressed the need for further research to explore the role of leukocytes in these diseases[1,2,4].

.***AIM***: In this study of 36 patients with CMPD, specific target genes Procoagulant TF, prothrombotic cytokines [IL1β,TNFα,] VEGF, Heparanase, Protease activated receptor2[PAR2] in clonal granulocytes were analyzed for disease associated aberrant gene expression involved in pathogenesis of haemostatic complications.

MATERIAL AND METHODS

Ethical approval was obtained from the ethical committee review board, Belfast City Hospital, Northern Ireland, U.K. and written informed consent was obtained from all subjects. Three groups-14 patients documented with PV [aged 44-76 years], 22 patients with ET [aged 31-81 years] and 18 healthy volunteers [aged 40-64 years] were recruited from the haematology department Belfast City Hospital. All documented patients with PV had elevated red cell mass, leukocytosis, thrombocytosis, with presence of clonal disease marker JAK2 V617F mutation, reduced serum erythropoietin and growth of BFU-E colonies. Documented ET patients were diagnosed by elevated platelet counts, normal serum ferritin level and presence of JAK2 V 617F mutation. Patients were considered ineligible for study if history of psychiatric disorder, pregnancy, lactation or oral contraceptive usage was present.

LABORATORY ANALYSIS

Venous blood granulocytes were obtained by Ficoll gradient ultracentrifugation.

Granulocyte lysates from the white cells pellet were prepared and stored frozen at -70'C. Total cellular RNA was isolated from granulocytes using an automated magnetic bead technology for RNA isolation employing the *MagNA Pure LC Instrument* with the

MagNA Pure LC RNA Isolation Kit-High Performance (Roche Diagnostics GmBH, Mannheim, Germany). Briefly 5x200µl of cell lysates[about 10^6 cells] were loaded and 50µl RNA was eluted from each sample and stored at – 70°C.

The amount of RNA was measured by spectrophotometry on the Thermo Scientific NanoDrop™ 1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA); the 260/280 and 260/230 absorbance ratios were used as a measure of the purity of RNA.

RT-PCR

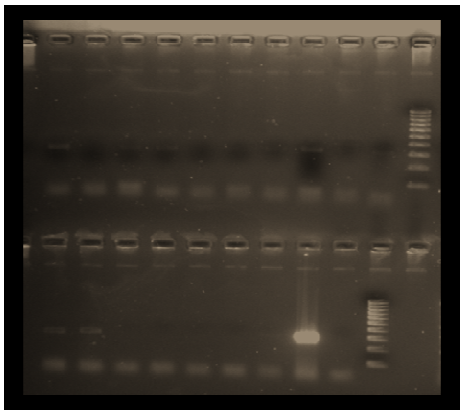
Reverse Transcription was carried out to obtain complementary DNA (cDNA) from participant RNA samples using the components of *Superscript First Strand Synthesis System* (Invitrogen, California, USA). Polymerase chain reaction was carried out with the cDNA as template, 45 µl of *Reddymix PCR Mastermix* (ABgene, Surrey, UK); 50pmol of forward primer, 50 pmol of reverse primer and 2 µl of cDNA were added and the reaction volume made to 50 µl with sterile water. PCR was performed with an initial incubation at 94°C for 2 minutes followed by 35 cycles of denaturation- annealing - extension with denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 1 minute. A final incubation was performed at 72°C for 5 minutes. Primer sequences used for PCR are shown in Table I. Final PCR products were subjected to agarose gel electrophoresis for 30 min. and qualitatively identified by UV transillumination photography.[Fig.1].

TABLE I
TARGET GENE PRIMER SEQUENCES USED FOR PCR

TARGET GENE	PRIMER	ORIENTATION	SEQUENCE	SPANS
TF	TF1F	Forward	ccgaacagttaaccggaaga	Exon5
TF	TF1R	Reverse	actcatttgcgtttccatgt	Exon6
HEPARANASE	HEP1F	Forward	tggacctggacttcttcacc	Exon2
HEPARANASE	HEP1R	Reverse	ttgattccttcttgggatcg	Exon3
TNFα	TNFαF	Forward	cgctccccaagaagacag	Exon1
TNFα	TNFαR	Reverse	tgggctacaggcttgtcact	Exon3
IL1β	IL1βF	Forward	accactacagcaagggcttc	Exon4
IL1β	IL1βR	Reverse	tccatatcctgtccctggag	Exon5
VEGF	VEGF1F	Forward	agccttgccttgctgctct	Exon1
VEGF	VEGF1R	Reverse	actccaggccctcgtcat	Exon3

FIGURE1
TF AMPLICONS IN ET-TISSUE FACTOR

1 2 3 4 5 6 7 8 9 10 M



11 12 13 14 15 16 17H C M

TISSUE FACTOR
LANES 1 TO 17 ET SUBJECTS
H HELA
C NO cDNA CONTROL
M 100 bp DNA LADDER

RESULTS

The average age of PV patients was 65 years [8 men,6 women],ET patients was 62 years[11 men,11 women] and healthy control subjects was 51 years[6 men,12 women]. The RNA yields were good and no extraneous DNA contamination in PCR products was present. The average RNA yield in the samples was 799.5 ng and ranged between 146.5ng and 2883ng. The 260/280 ratio varied from 1.18 to 2.63 (mean 1.93) while the 260/230 ratio was 0.87 on an average (range- 0.35 to 1.87).

RTPCR results were used to determine the presence or absence of expression of each target gene. Expression of each target gene was decided upon by two criteria- the presence of a band of predicted size upon agarose gel electrophoresis of the PCR products, and, the band was clearly visible on UV transillumination. The RT PCR results of 5 target genes in agarose gel electrophoresis is summarized in Table II.

TF was expressed by 43% of PV patients and 27% of ET patients whereas none of the controls expressed TF. Heparanase was expressed by 85% PV, 73% of ET and 72% of controls. Expression of TNFα, IL1β and VEGF was seen in almost every patient and control.

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TABLE II
RTPCR Results in PV and ET
Detection of Target Gene Expression (%Total in each group in parenthesis)

TARGET GENE	PV(n=14)	ET(n=22)	N(n=18)
TF	6(42.9)	6(27.3)	0(0)
HEPARANASE	12(85.7)	16(72.7)	13(72.2)
TNFα	14(100)	22(100)	16(89.1)
IL1β	14(100)	22(100)	17(94.4)
VEGF	14(100)	22(100)	18(100)

DISCUSSION

Haemostasis-related genes in granulocytes of MPD were studied for aberrant expression in PV and ET. TF is the physiological activator of coagulation. Studies have shown that polymorphonuclear cells, monocytes and platelets express TF at low levels and the expression is inducible by various stimuli[5]. Several reports suggest that the expression of TF is induced by the prothrombotic cytokines, PAR2 and heparanase[6]. PAR2 is a cellular receptor for TF/Factor VIIa complex. Heparanase, an endo-glucuronidase widely expressed by tumor cells, was reported to induce TF expression in acute leukaemias. The cytokines TNFα, IL1β and VEGF are upregulated in prothrombotic diseases including cancers, atrial fibrillation and antiphospholipid antibody syndrome[7]. IL1β-mediated TF upregulation was found to contribute to thrombosis in disseminated intravascular coagulation[8,9]. Thus the present study was designed to evaluate the expression of these haemostasis-related genes in MPD seeking any disease-associated changes.

Qualitative analysis of TF expression in our study yielded interesting results. None of our control subjects were found to express TF. This was in accordance with the findings of Maugeri *et al* who reported that PMN cells from healthy volunteers do not constitutively express TF but only upon stimulation[5]. In subsequent studies it was found that PMN leukocytes of MPD patients had significantly higher levels of TF antigen even in the resting state. In our study TF was found to be expressed at low levels in <50% of patients as indicated by the faint bands on agarose gel electrophoresis of RT-PCR products. Quantification of granulocyte TF expression would give a better picture of TF expression by PMN cells and the disease-related changes in expression if any. The results of RT-PCR for Heparanase, VEGF, TNFα and IL1β revealed no specific patterns of expression as each was found to be expressed in virtually all PV and ET patients and all control subjects. RT-PCR is an end point analysis and thus these results do not necessarily indicate absence of disease-related differences in expression. It is important to acknowledge the small sample size in our study while interpreting our results. Thus the absence of significant differences in expression could be due to the limited number of subjects analyzed and need not necessarily indicate true absence of any difference. A larger sample size would, possibly, help to detect significant differences in expression, if any, between MPD patients and control subjects. This 'proof of concept' study is intended to serve as a

guide for future studies to explore the relationship between the expression of these genes and the occurrence of haemostatic complications in MPD patients.

CONCLUSION

Upregulation of TF and other haemostasis related genes in granulocytes genes as suggested by this qualitative method of RTPCR suggests altered gene expression in granulocytes of MPD patients. This could be further explored by quantitative studies.

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