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THE ROLE OF FASR/FASL SYSTEM IN PATHOGENESIS OF MYELOPROLYFERATIVE NEOPLASMS

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Abstract - Myeloproliferative neoplasms (MPN) are hematological malignancies characterized by uncontrolled cell proliferation and impaired apoptosis. The FasR/FasL system is involved in the control of apoptosis in different cell types. Here we have investigated the role of FasR/FasL in the pathogenesis of MPNs. We compared FasR/FasL expression between MPN patients (24) and healthy individuals using the real-time PCR assay. We found an increase of FasR expression in MPN patients. No difference was detected in FasL expression. Mutation V617F in the *JAK2* gene, a hallmark of MPN, was detected in 13/24 patients. We found that neither FasR nor FasL expression were related to the presence of *JAK2* V617F mutation.

Key words: Myeloproliferative neoplasms, FasR/FasL, *JAK2* V617F mutation

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INTRODUCTION

The number of cells in the body is strictly regulated and tightly controlled to maintain normal tissue homeostasis. Different mechanisms are enrolled in the control of cell survival and cell death. Apoptosis is one of these mechanisms, maintaining cellular homeostasis in a variety of tissues, particularly in rapidly renewing ones, such as hematopoietic tissue. It has an important role during embryogenesis and tissue remodeling, and plays a central role in immune system response. Reduced apoptosis can contribute to the pathogenesis of cancer, autoimmune disorders and sustained viral infections. On the other hand, excessive apoptosis results in inadequate cell loss and consequent degenerative disorders, such as, Alzheimer disease (Thompson, 1995).

Cell survival and cell death are controlled by a finely tuned ensemble of pro-survival and pro-apoptotic proteins. One of them is the Fas receptor (FasR) also known as CD95/Apo-1, that belongs to the tumor necrosis factor (TNF-R) receptor family. It contains an intracellular "death domain" and is present on the cell surface as a monomer protein. Its natural ligand, Fas ligand (FasL/CD178), is a member of the TNF cytokine family. It is present as a trimeric protein on the cell surface. It has been shown in both murine and cell culture studies that the critical role of FasR is in the immune system, where it is involved in provoking the apoptosis of pathogen-infected cells, preventing the excretion of its content in extracellular space. In this way it functions as a guardian against autoimmunity and tumor development (Strasser et al., 2009).

Myeloproliferative neoplasms (MPNs) form a range of clonal hematological malignant diseases, the members of which are polycythaemia vera (PV), essential thrombocythaemia (ET) and idiopathic myelofibrosis (IMF) (Baxter et al., 2005). The myeloproliferative neoplasms are characterized by overactive hematopoiesis, with increased production of red blood cells and platelets as a major feature of PV and ET, respectively (Baxter et al., 2005). An immanent characteristic of these diseases is the proliferation of certain cell types independent of cytokine stimula-

tion. Another common feature of these diseases is acquired somatic mutation V617F in the *JAK2* gene, which is present in 97% of patients with PV, and half of the patients with ET and IMF. This mutation is characterized by G>T transversion at position 1849 in exon 14 of the *JAK2* gene leading to a substitution of valine by phenylalane at position 617 of the JAK2 protein (Baxter et al., 2005; James et al., 2005; Jones et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Zhao et al., 2005). This mutation is located in the JH2 pseudokinase domain of the JAK2 protein, which is involved in auto-inhibition of its kinase activity. This nucleotide exchange leads to the constitutive activation of the *JAK2* gene (Kralovics et al., 2005). Consequently, many transduction pathways that are regulated by the JAK2 protein are altered as well.

The significant role of the FasR/FasL system has been well defined and characterized in detail in the immune system, but deregulation of FasR or FasL in other hematopoietic cell compartments is not yet well defined. Granulocytes express FasR on their surface and antibody- or ligand-mediated activation of receptors triggers their apoptotic death (Hebestreit et al., 1996; Liles et al., 1996). Erythrocytes in different stages of their development express FasR and FasL in different levels and the binding of the ligand leads either to apoptosis or maturation block (Strasser et al., 2009). FasR is expressed on the majority of human leukemia cells, although the expression level is variable (Komada et al., 1995).

In this paper we present our findings regarding the enrollment of the FasR/FasL system in the pathogenesis of myeloproliferative neoplasms. For this purpose 24 MPN patients and 6 healthy controls were analyzed for the expression of Fas receptor and Fas ligand using real-time PCR methodology. Consequent detection of the V617F *JAK2* mutation was performed to determine whether the Fas apoptotic pathway contributes independently to the malignant phenotype of myeloproliferative neoplasms, or correlates with the presence of the JAK2 mutation.

MATERIALS AND METHODS

Subjects

Peripheral blood samples were obtained from 24 patients with myeloproliferative neoplasms. The patients were diagnosed with PV (14) and ET (10) according to World Health Organization diagnostic criteria (Tefferi and Vardiman, 2008). They were further referred to the Institute of Molecular Genetics and Genetic Engineering in Belgrade, for V617F *JAK2* analysis. Peripheral blood samples from 6 healthy donors have also been included in this study. All patients gave informed consent.

RNA extraction and cDNA synthesis

Total RNA was obtained from peripheral blood mononuclear cells using a TRIzol reagent (Invitrogen, USA) according to manufacturers` instructions. Reverse transcription was performed using the Europe against Cancer Group (EAC) protocol. Briefly, 2 μg of total RNA and 25 μM random hexamers (Fermentas, Lithuania) in 11 μl were incubated at 75˚C for 5 min and cooled on ice. Then other reagents were added to a final volume of 20 μL, including 200 U M-MuLV Reverse Transcriptase (Fermentas, Lithuania), 5xRT buffer, dNTPs 1mM and 20 U RNase inhibitor (Fermentas, Lithuania). The mixture was incubated for 5 min at 25ºC prior to the addition of M-MuLV RT, and then incubated 60 min at 42ºC, followed by 10 min at 72ºC and 10 min at 4ºC. Aliquots were stored at -80ºC until further analysis.

Quantification of FasR and FasL expression

Quantitative real-time PCR (RQ-PCR) for FasR and FasL expression was performed using the TaqMan chemistry in a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). L13/high basic protein gene (HBP), which encodes for a protein of a large ribosome subunit, was used as an endogenous control (Fu et al., 2001).

The final volume of the RQ-PCR reaction was 10 μl. It included cDNA (40 ng RNA equivalent), primers

400 nM each (for FasR and FasL), 200 nM, for HBP, each probe 300 nM, and TaqMan Universal PCR master mix 5 μl. The cycling conditions were as follows: 10 min at 95˚C, followed by 50 cycles at 95˚C for 15 s, 60˚C for 1 min.

The primers and probe for FasR were 5'-TGA AGG ACA TGG CTT AGA AGT G-3'(FasR-fwd), 5'- GGT GCA AGG GTC ACA GTG TT-3'(FasR-rev), 5'-(FAM)-AAA CTG CAC CCG GAC CCA GAA TAC C-(TAMRA)-3'(FasR probe), producing 118 BP PCR amplicon. For FasL the primers were 5'-GCA GCC CTT CAA TTA CCC AT-3'(FasL-fwd), 5'-CAG AGG TTG GAC AGG GAA GAA-3'(FasL-rev), and probe 5'-(FAM)-TCC CCA GAT CTA CTG GGT GGA CAG C-(TAMRA)-3'(FasL probe), producing 101 BP PCR amplicon. For the endogenous control (HBP) primers and probe were as follows; 5'-ACC GGT AGT GGA TCT TGG CTT T-3'(HBP-fwd), 5'-GCT GGA AGT ACC AGG CAG TGA-3'(HBP-rev), 5'-(VIC)-TCT TTC CTC TTC TCC TCC AGG GTG GCT-(TAMRA)-3'(HBP probe), resulting in a 104 bp PCR amplicon.

We analyzed the 24 MPN samples and the 6 samples collected from the healthy donors (control group). All of the samples were analyzed in duplicate.

The cycle number at which the reaction crossed an arbitrarily placed threshold (Ct=0, 1, baseline [3, 15]) was determined for each sample and for each gene (FasR, FasL, HBP) using 7500 System software (version 1.3.1.) (Applied Biosystems, Foster City, CA, USA).

REST® software (Relative Expression Software Tool 2008) was used to analyze the difference in the gene expression between the two groups of samples (Pfaffl et al., 2002, http://rest.gene-quantification.info). In this software the relative quantification of a target gene is based on the mean Ct deviation of the control and sample group, normalized by a reference gene. Then, the difference in gene expression between the two groups is tested for significance by a randomization test. The obtained P value represents the probability of the alternate hypothesis, that the difference between the two groups is one of chance only.

Allele-specific PCR for the detection of JAK2–V617F mutation

The *JAK2*–V617F mutation was detected according to the protocol of Baxter et al., (2005) with some modifications. We isolated peripheral blood granulocytes on a Ficoll gradient (SIGMA Aldrich) according to the manufacturer's instructions. Genomic DNA was extracted from granulocytes using the QIAampDNA BloodMini Kit (Qiagen). For each sample 80 ng of each patient's DNA was amplified in PCR reaction. The primers used for PCR were:

Fcont:
5'ATCTATAGTCATGCTGAAAGTAGGAGAAAG3'

Fspec:
5'AGCATTTGGTTTTAAATTATGGAGTATATT3'

Reverse:
5'CTGAATAGTCCTACAGTGTTTTCAGTTTCA3'

Fcont primer with a common reverse primer generates a 358-bp product from both mutant and wild-type alleles. Fspec forward primer with a common reverse primer generates a 197-bp product. The first PCR round was done with 1 μmol/L of each of the Fcont and R primers, and a second round was performed with 1 μmol/L Fspec and R primers. QIAGEN Hot start polymerase was used in all reactions. The conditions of the first PCR were: 15 min at 95ºC followed by 35 cycles (94ºC for 30s, 58ºC for 30s, 72ºC for 30s), and 10 min at 72ºC after the last cycle. For the second round the annealing temperature was 62ºC. The PCR products were analyzed on 2% TAE agarose gels.

The same procedure was used to detect *JAK2* - V617F mutation in mononuclear cells of the MPN patients.

Direct sequencing of a PCR product

Genomic DNA was amplified using Fcont and Reverse primers. The PCR product was directly

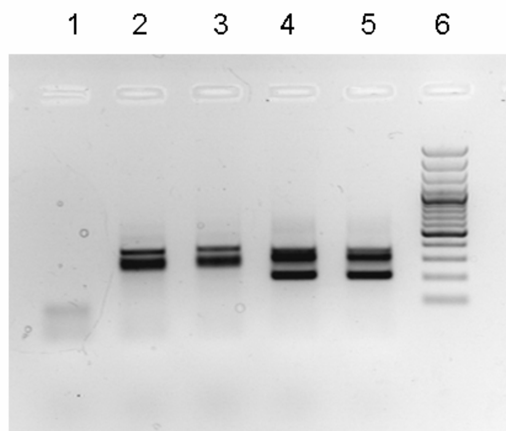

Fig. 1. Detection of *JAK2*-V617F mutation by allele-specific PCR. 1. Water control; 2, 3. *JAK2*-V617F-negative patients; 4, 5. *JAK2*-V617F-positive patients; 6. DNA marker 100 bp ladder

sequenced using reverse primer on an ABI 3700 machine by BigDye terminator sequencing (Applied Biosystems, Foster City, CA, USA). For sequencing we used a reverse primer because sequencing with a forward primer resulted in unresolved sequence chromatograms probably due to the presence of T-stretch 5' of the mutation. The chromatogram was analyzed using Sequencing analysis 5.2 (Applied Biosystem) software.

RESULTS

Analysis of Fas receptor and Fas ligand expression in MPN patients

We have analyzed samples from 24 MPN patients and 6 healthy controls using real-time PCR assay. Statistical analysis of FasR showed increased expression in the MPN patients compared to the healthy controls and this difference was statistically significant (Table 1). For all tests performed, the number of randomizations was 2000; 95% C.I. for FasR was within the range 0, 11-11.546, 806; P (H1) 0,034; standard error 0,215-1.608, 323.

The analysis of expression of Fas ligand in our study group of MPN patients showed no difference compared with the healthy individuals (95% C.I. for FasL was within the range 0,003-96,594; P (H1) 0,833; standard error 0,029-25,711 (Table 2).

Detection of JAK2 V617F mutation

Consequent detection of the V617F *JAK2* mutation was performed to determine if the Fas apoptotic pathway contributes independently to the malignant phenotype of myeloproliferative neoplasms or correlates with the presence of the JAK2 mutation.

Using allele-specific PCR for the detection of V617F *JAK2* mutation in the peripheral blood granulocytes of MPN patients (Figure 1), we found that 13 patients were positive for the mutation and 11 patients were negative. The mutation was also detected on the mononuclear cells for all the patients that showed a positive mutational status on the peripheral blood granulocytes. In order to confirm the results from allele-specific PCR, the PCR products were subsequently sequenced. The V617F *JAK2* mutational status was fully confirmed for all analyzed samples.

Analysis of Fas receptor and Fas ligand expression in MPN patients in relation to the presence of V617F JAK2 mutation

Comparison of FasR expression between the samples of patients harboring V617F *JAK2* mutation and patients without the mutation, showed no statistically significant difference (data not shown). There was also no difference in FasL expression in these two groups of patients (data not shown).

DISCUSSION

Apoptosis is triggered with two pathways: intrinsic and extrinsic. The intrinsic apoptotic pathway is activated in response to insufficient trophic support (growth factor withdrawal), exposure to anti-tumor drugs and UV-radiation. The extrinsic apoptotic pathway (death-receptor pathway) is activated upon interaction of the death receptor with its cognate ligand (Testa and Riccioni, 2007). Growing evidence indicates that the extrinsic apoptotic path-

Table 1. Analysis of Fas receptor expression in MPN patients

Gene	Type	Reaction efficiency	Expression	St. Error	95% C. I.	P(H1)	Result
HBP	Ref	0.9371	1.000				
FasR	Trg	0.8727	17.666	0.215-1.608,323	0.011-11.546,806	0.034	upregulated

Legend:
P(H1) – probability of alternate hypothesis that difference between sample and control group is due only to chance
Trg – target
Ref – reference
C. I.- confidence interval

Table 2. Analysis of Fas ligand expression in MPN patients

Gene	Type	Reaction efficiency	Expression	St. Error	95% C. I.	P(H1)	Result
HBP	Ref	0.9371	1.000				
FasR	Trg	0.65	0.780	0.029-25.711	0.003-96.549	0.833	not different

Legend:
P(H1) – probability of alternate hypothesis that difference between sample and control group is due only to chance
Trg – target
Ref – reference
C. I.- confidence interval

way, especially the FasR/FasL system, could play a relevant role in hematopoiesis, regulating cell number and differentiation of blood cells. The FasR protein belongs to the tumor necrosis factor receptor (TNF-R) family and its ligand, FasL, is a member of the TNF cytokine family. When FasL binds to FasR located on an adjacent cell, it causes the trimerization of the FasR death domain. This event can also be mimicked by the binding of an agonistic Fas antibody (Nagata, 1999). Ligation of FasR rapidly causes the assembly of an intracellular "death-inducing signaling complex" (DISC) (Kischkel et al., 1995). DISC contains the aspartate-specific cysteine protease, caspase-8, its adaptor/activator FADD and its modulator, c-FLIP (Irmler et al., 1997). FADD-mediated activation of the proteolytic activity of caspase-8 is essential for Fas-induced apoptosis in the majority of cell types (including lymphoid and other hematopoietic ones) that were investigated, both *in vitro* and *in vivo* (Kang et al., 2004; Kang et al., 2008).

Myeloproliferative neoplasms comprise three different entities of hematological malignancies: polycythaemia vera (PV), essential thrombocythaemia (ET) and idiopathic myelofibrosis (IMF). In all these diseases myeloid cell lineage is affected, suggesting that the mutation event, relevant for the pathogenesis of these diseases, occurs in the common myeloid progenitor cell (Jones et al., 2005). Differentiated myeloid cells, as well as progenitor cells, express FasR/FasL on their surfaces. Moreover, activated platelets express FasL and induce apoptosis in Fas-positive tumor cells (Ahmad et al., 2001). Also, granulocytes express FasR on their surface and antibody- or ligand-mediated activation of receptors triggers their apoptotic death (Hebestreit et al., 1996; Liles et al., 1996). In addition, a recently proposed model for the control of erythropoiesis suggests that the TNF family ligands expressed by mature erythroid precursors can interact with their cognate receptors expressed on the surfa-

ces of immature erythroblasts (De Maria et al., 1999; Zamai et al., 2000; Felli et al., 2005).

The intrinsic apoptotic pathway is affected in MPNs directly through the JAK-STAT signaling pathway. Namely, activating somatic mutation V617F in the *JAK2* gene, which is usually present in these patients, causes sustained phosphorylation of STAT5, Akt/PKB, and ERK kinases in the absence of cytokine stimulation. A downstream target of STAT5 protein is the Bcl-xL antiapoptotic protein, a member of the Bcl2 family of pro-apoptotic and pro-survival proteins (Socolovsky et al., 1999; Dumon et al., 1999; Ariyoshi et al., 2000). Bcl-xL activation suppresses the apoptosis in MPN patients and its overexpression contributes to erythropoietin-independent survival of erythroid-lineage cells in polycythaemia vera patients (Silva et al., 1998). In this way JAK2 mutation directly contributes not only to increased proliferation, but also to decreased apoptosis in these patients.

Nevertheless, the extrinsic apoptotic pathway is also deregulated in MPNs. It was shown that the activation of Fas receptors on the surfaces of immature erythroblasts in healthy individuals results in a reversible arrest of proliferation and differentiation or in cell death. Interestingly, erythroblasts from polycythaemia vera patients showed significant resistance to Fas-mediated growth inhibition, and also showed lower apoptotic rate (Zeuner et al., 2006). For the erythroid lineage, the effect of Fas receptor stimulation on caspase-8 activation and cleavage of the GATA-1 transcription factor is probably the most important event. It can be speculated that the lower caspase-8 activation observed in PV patients causes ineffective cleavage of GATA-1, contributing to the survival and growth of these cells in PV patients (Zeuner et al., 2006).

Here we demonstrate increased FasR expression in two MPN entities: polycythaemia vera and essential trombocythaemia. Our study group enrolled 24 MPN patients, 14 with polycythaemia vera and 10 with essential thrombocythaemia. Our findings contribute to previous results, strongly suggesting that the extrinsic apoptotic pathway is affected in MPN patients via upregulation of FaR expression, while the FasL expression was unaffected.

It was also interesting to look for a possible correlation between the presence of JAK2 mutation and the upregulation of FasR in these patients. To address this question, we compared two groups of patients: one, harboring the V617F *JAK2* mutation, with the other, V617F *JAK2* negative. This comparison showed that there is no difference in FasR or FasL expression between these two groups, suggesting that the Fas apoptotic pathway independently contributes to the phenotype of MPNs.

Noticeable variations of FasR and FasL expression in patients as well as in healthy individuals have been observed. This is probably due to the unhomogenic population of cells used in these experiments. It could be of interest to sequester cell populations and study this phenomenon on separated cell populations.

Further investigation of the significance of FasR in MPNs, and its relationship with the intrinsic apoptotic pathway is needed. Interactions between these two pathways could better elucidate the pathogenesis of myeloproliferative neoplasms.

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УЛОГА СИСТЕМА FASR/FASL У ПАТОГЕНЕЗИ МИЈЕЛОПРОЛИФЕРАТИВНИХ НЕОПЛАЗИЈА

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Мијелопролиферативне неоплазије (MPN) су хематолоши малигнитети који се карактеришу неконтролисаном ћелијском пролиферацијом и поремећајем у процесу апоптозе. Систем FasR/FasL је укључен у контролу апоптозе у различитим типовима ћелија. У овом раду је изучавана улога система FasR/FasL у патогенези мијелопролиферативних неоплазија. Упоређена је експресија FasR и FasL између пацијената са MPN (24) и здравих контрола коришћењем методе „real-time“ PCR. Детектована је повећана експресија FasR код пацијената са MPN. Није утврђена разлика у експресији FasL. Мутација B617F у *JAK2* гену, карактеристична за MPN, је нађена код 13 од 24 пацијента. Показано је да експресија FasR и FasL није повезана са присуством B617F *JAK2* мутације.