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IMMUNOCYTOCHEMICAL STUDY OF BCR AND BCR-ABL LOCALIZATION IN K562 CELLS

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Aim: **To obtain polyclonal antibodies against recombinant proteins recognizing Bcr domain and fusion region of Bcr-Abl and analyze the patterns of intracellular distribution of Bcr and Bcr-Abl proteins in K562 cells of chronic myelogenous leukemia.** ***Methods:*** **The coding sequences of DH and PH domains of Bcr-Abl were cloned, and the recombinant proteins were expressed in *E. coli*. The rabbit polyclonal antibodies were produced and used for immunocytochemical study of Bcr and Bcr-Abl localization in K562 cells.** ***Results:*** **The gene constructs containing sequences coding for DH and PH domains of Bcr-Abl have been obtained. The antibodies with relative specificity to corresponding recombinant proteins differ by the patterns of their intracellular reactivity with Bcr- and Bcr-Abl related structures. While Bcr protein is located predominantly perinuclearly, antibody against hybrid Bcr-Abl protein is reacted with the structures in cell periphery, namely on cell membranes.** ***Conclusion:*** **Antibodies against DH and PH domains of Bcr-Abl react with proteins located differently in chronic myelogenous leukemia cells. The difference in intracellular localization of Bcr and Bcr-Abl may be attributable to the different domains interacting with different multiprotein complexes.**
Key Words: **Philadelphia chromosome, Bcr-Abl, CML, ALL, polyclonal antibodies, K562 cells.**

Bcr-Abl, a constitutively active cytoplasmic tyrosine kinase essential for the initiation of chronic myelogenous leukemia (CML) is coded by the gene derived from the fusion of the breakpoint cluster region (*BCR*) gene on chromosome 22 and the Abelson leukemia oncogene (*ABL*) on chromosome 9. At diagnosis, up to 95% of CML cases have the characteristic t(9; 22)(q34; q112) reciprocal translocation giving the origin to Philadelphia chromosome [1]. Depending on the breakpoint region of the *BCR* gene implicated in the translocation, various Bcr-Abl chimeras have been observed: p190 Bcr-Abl, p210 Bcr-Abl and p230 Bcr-Abl [2]. The most frequent one is p210 Bcr-Abl, which is responsible for CML, while p190 Bcr-Abl is responsible for acute lymphoblastic leukemia (ALL). Rarely, the breakpoint in *BCR* gene occurs in μ-BCR region spanning exons 17–20 and a larger fusion protein p230 is encoded. Patients with this fusion demonstrate prominent neutrophil maturation and/or conspicuous thrombocytosis.

The only structural difference between p190 and p210 Bcr-Abl is the presence of Dbl homology (DH) and pleckstrin homology (PH) domains in p210 Bcr-Abl. Thus, a complete understanding of the biological mechanisms underlying the origin of ALL and CML requires the characterization of the signaling activities that reside within Bcr. The isolated recombinant DH domain of Bcr is suggested to be an activator of Rho GTPases [3]. The function of PH domain has not yet been clarified in details. Recently, PH domain has been shown to bind various phospholipids and to take part in protein-protein interactions [4]. The resulting Bcr-Abl fusion protein acts as an oncoprotein and the constitutive activation of tyrosine kinase activity contributes to Bcr-Abl mediated leukemogenesis. p210 Bcr-Abl unlike normal p145 c-Abl has been shown to localize predominantly in cytoplasm [5]. The ectopic expression of p210 Bcr-Abl seems to be a factor contributing to leukemic transformation of hematopoietic cells. In the cytoplasm Bcr-Abl interacts with multiple signal transduction pathways that transmit anti-apoptotic and mitogenic signals. The key pathways involve Ras, MAP kinases, the STAT family, PI3 kinase, and myc, among others [2, 6].

The aim of the study is to obtain the specific antibodies recognizing Bcr domain and fusion region of Bcr-Abl and to study immunocytochemically the patterns of intracellular distribution of Bcr and Bcr-Abl proteins in the cells of chronic myelogenous leukemia.

In this study, the gene constructs containing sequences coding for DH and PH domains of Bcr-Abl have been obtained, the corresponding fusion proteins were produced in bacteria, purified and used for the immunization. The polyclonal antibodies to Bcr domain and to region of Bcr-Abl fusion were obtained and the subcellular localization of Bcr-Abl and Bcr in K562 cells was examined. Our data should help us to understand the molecular mechanisms underlying the phenotypes of Bcr-Abl positive leukemias and to find new targets for therapeutic intervention.

MATERIALS AND METHODS

Cell line. Bcr-Abl positive K562 cells of blast phase of human chronic myelogenous leukemia were obtained from cell line depository of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine. The cells were cultured in suspension in 5% CO_2 — 95% air mixture at the temperature of 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 0.1% gentamycin (Hyclone), and 1% L-glutamine (Hyclone).

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Abbreviations used: ABL – Abelson leukemia oncogene; BCR – breakpoint cluster region; CML – chronic myelogenous leukemia.

Cloning of coding sequences of DH and PH domains of BCR-ABL. Expression construct pDDBA3 based on *pET32b* vector coding for DH domain of Bcr was designed in the following way: The amplification product of cDNA corresponding to the region 1955–2810 b.p. according to GenBank X02596.1 was cloned in pET32b at EcoRI site. The recombinant protein was expressed in BL21/DE3 strain of *E. coli*. Another expression construct pPDBA 138 designed on the basis of *pET32b* vector was used to obtain the recombinant protein comprising PH domain of Bcr and Abl fragment corresponding to 2744–3333 b.p. region of Bcr-Abl according to GenBank X02596.1. The details of cloning procedures are presented in [3]. The alignment of the coding sequences in both constructs is schematically given in Fig. 1.

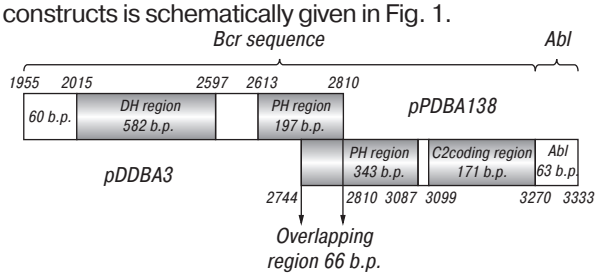

Fig. 1. Schematical alignment of the cloned fragments of *Bcr-Abl* in expression constructs *pDDBA3* and *pPDBA138*

Purification of recombinant proteins and production of polyclonal antibodies. The recombinant proteins DDBA3 and PDBA 138 were purified by affinity chromatography on Ni-NTA agarose and dialyzed against PBS pH 7.4. The purity of antibodies was assessed by electrophoresis in agarose gel (Fig. 2).

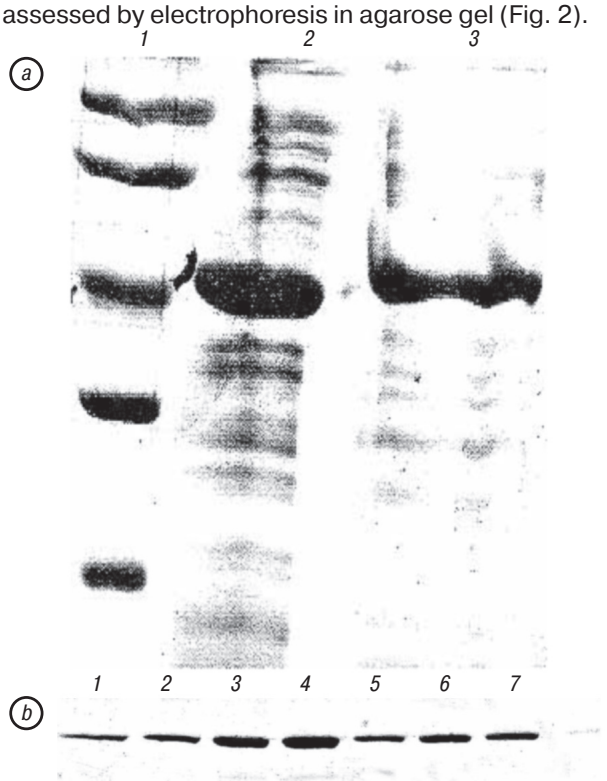

Fig. 2. Electrophoregrams of purification stages of recombinant protein PDBA138: *a*, 1 — LMW standards; 2 — purified inclusion bodies; 3 — soluble fraction of recombinant protein PDBA138 after dialysis against PBS; *b*, isolation of recombinant proteins by affinity chromatography under the native conditions on Ni-NTA agarose: 1–7 stages of elution with step-wise imidasol gradient 40, 60, 80, 100, 150, 200, 250 mM

The purified proteins were used for producing polyclonal antibodies. The recombinant proteins mixed with complete Freund adjuvant were injected to the male rabbits, and in 8 and 12 weeks booster immunizations followed. The titer of specific antibodies was assessed in serum by immunoenzyme technique. The antibodies were depleted on BrCN-activated Sepharose (Amersham Pharmacia Biotech Inc., USA) with immobilized total bacterial proteins of *E. coli* BL21(DE3) as well as the peptide expressed by pET32 itself. The specificity of the antibodies obtained was assessed by Western blot.

Immunochemical technique. K562 cells were used as a model for immunocytochemical study of Bcr and Bcr-Abl localization. The cells were fixed with formalin-acetone pH 7.6 and permeabilized with 0.1% Triton X-100. The specimens were treated with anti-DDBA3 or anti-PDBA138 antibodies. EnVision^{AP} system (DAKO, Denmark) was used as the secondary antibody. The immunochemical staining was visualized upon cytochemical reaction with naphtol-AS-BI-phosphate as the substrate of alkaline phosphatase.

RESULTS AND DISCUSSION

The results of Western blot with purified antibodies against DDBA3 and PDBA 138 proteins are given in Fig. 3. The lysates from K562 cells, the peripheral blood cells of CML patients and the healthy donors were used. Both anti-DDBA3 and anti-PDBA 138 reveal p210 and p160 proteins in K562 cells and in cells from CML patients. The cross-reactivity may be explained by the presence of the short overlapping Bcr sequence, 22 amino acid residues in length (see Fig. 1), in both recombinant proteins. Nevertheless, the affinity of binding seems to be more pronounced in the case of anti-PDBA 138 antibody. Therefore, we attempted to use both antibodies in the analysis of intracellular distribution Bcr and Bcr-Abl in K562 cells.

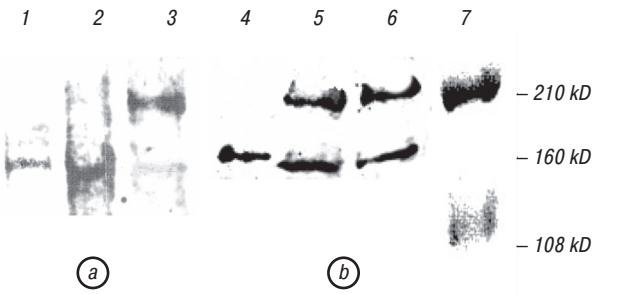

Fig. 3. Western blot with polyclonal anti-DDBA3 and anti-PDBA138 antibodies against the corresponding recombinant proteins. *a, b* — detection of chimerical protein Bcr-Abl (210 kD) and its normal analogue (160 kD) in polymorphonuclear cells of healthy donors and CML patients with polyclonal anti-DDBA3 (*a*) and anti-PDBA138 (*b*) antibodies: 1, 4 — healthy donors; 2, 3, 6 — CML patients; 5 — K562 cells; 7 — HMW protein standard (Sigma, USA)

The polyclonal antibodies against DDBA3 and PDBA 138 proteins recognizing different regions of Bcr-Abl hybrid protein allows one to analyze the intracellular location of Bcr-Abl as well as Bcr proteins in CML cells *in vivo*. The results of immunocytochemical analysis of K562 cells with antibodies against DDBA3 and PDBA 138 proteins are presented in Fig. 4. Bcr protein is located predominantly perinuclearly, while anti-PDBA138 antibody against

hybrid Bcr-Abl protein is revealed predominantly in cell periphery, namely on cell membranes. Therefore, the anti-DDBA3 and anti-PDBA138 antibodies with relative specificity to corresponding recombinant proteins differ by the patterns of intracellular reactivity with Bcr- and Bcr-Abl related structures.

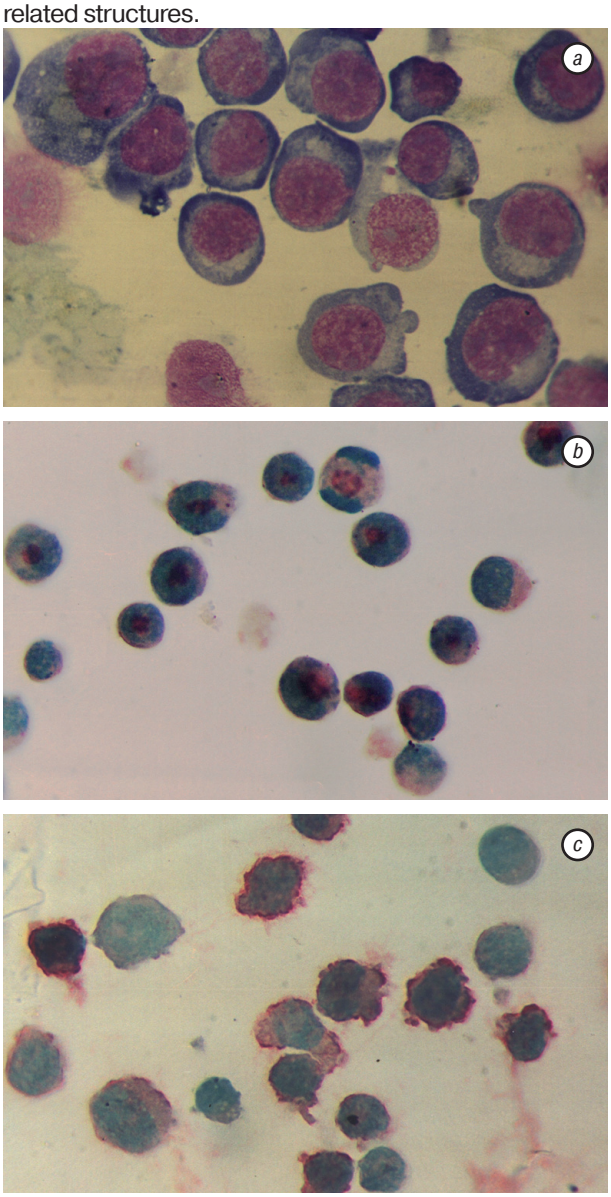

Fig. 4. Topography of Bcr and Bcr-Abl in K562 cells assessed with polyclonal anti-DDBA3 and anti-PDBA138 antibodies against the corresponding recombinant proteins: *a* — K562 cells, MGG staining, x 900; *b* — perinuclear pattern of Bcr distribution (anti-DDBA3); *c* — membrane pattern of Bcr-Abl distribution (anti-PDBA138)

In K562 cells transformed with pEGFP-Bcr-Abl construct, the protein has predominantly cytoplasmic localization generating cortical-F-actin ring [7] due to actin-binding domain in Abl moiety of Bcr-Abl hybrid protein [3, 8]. Also, in 32D myeloid cells Bcr-Abl locates on vesicle-like structures that lacked detectable F-actin [9]. Such binding is not affected by mutations in actin-binding domain. Our previous data [10] seem to hint at the mechanisms of such location. Namely, PH domain has been shown to bind to the membrane of Golgi complex, in particular with $PIP_{(3)}$ and $PIP_{(4)}$. The different patterns of the intracellular localization of Bcr and Bcr-Abl may be attributable to the different domains interacting with different multiprotein complexes. PDZ-binding domain in C-terminal part of Bcr provides for its interaction with AF-6 protein facilitating Ras binding producing the multimeric complex. As a result, Ras and ERK are down-regulated [11]. Besides, PDZ-binding domain provides for the interaction between Bcr and the apical PDZ-K-1and Mint 3, the latter being the component of the vesicular trafficking in the secretory pathway [12]. For understanding the role of Bcr in cell biology, the fact of its displacement to the membrane upon the effects of growth factors is of high importance [13].

Thus, our data show that in K562 CML cells Bcr-Abl is predominantly localized to the cell periphery. This fact seems to be explained by the presence of actin-binding domain in Bcr-Abl hybrid protein. On the other hand, the localization of Bcr may be partly explained by the presence of PH and PDZ-binding domains.

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