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Original Article

Preclinical model for identification of therapeutic targets for CML offers clues for handling imatinib resistance

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ABSTRACT

Success of imatinib in chronic myeloid leukemia (CML) therapy has undoubtedly proved utility of signalling molecules as therapeutic targets. However, development of imatinib resistance and progression to blastic crisis are the current challenges in clinics. To develop therapeutic alternatives for CML, understanding of signalling events downstream of bcr-abl might be helpful. Current CML cell lines do not give comprehensive picture of signalling events involved in pathogenesis of CML. Hence, there is a major unmet need for a better preclinical model for CML. Here, we report on development of RIN9815/bcr-abl, a novel cell line model that mimics signalling events in CML PMNL. Studies on crucial signalling molecules i.e., ras, rac, rhoA and actin in this cell line identified rhoA as the key regulator involved in CML cell function as well as proliferation of both, imatinib sensitive and resistant cells. Hence, RIN9815/bcr-abl could serve as the unique preclinical model in understanding pathogenesis of CML and in drug development.

1. Introduction

Ph[1] chromosome, a hallmark of CML encodes for bcr-abl gene that translates a protein p210, with high and unregulated tyrosine kinase activity [1]. Although much has been learnt about the biology of bcr-abl still a complete cure of CML is not achieved. Bcr-abl protein, p210 has the ability to transform various haemato-poietic and non-haematopoietic cells. Transformation by bcr-abl leads to activation of various signalling pathways involved in cell proliferation and morphology, such as ras, MAPK, JNK/SARK, PI3K, NF-kB and STAT. Several of these pathways contribute to cellular transformation. In view of success of bcr-abl kinase inhibitor – imatinib in CML therapy, understanding of downstream signalling events may help in development of alternative, promising therapeutic approaches.

PMNL, the terminally differentiated myeloid cells from CML patients exhibit defects in several actin dependent functions, such as chemotaxis, adhesion, phagocytosis and microbicidal activities, due to global defects in actin polymerization [2]. Ras, which is one of the bcr-abl targets, is the key signaling molecule in actin polymerization pathway. Retention of the actin-binding domain of bcr-abl enhances its transforming activity. In view of the role of actin in cell division and PMNL functions, the ras-mediated actin polymerization pathway may be relevant in leukomogenesis. Our studies on signalling molecules involved in actin polymerization have identified rhoA as the key molecule responsible for functional defects in CML PMNL [3]. It is difficult to study actin polymeriza-tion in CML cell lines due to lack of surface receptors for classical stimulants inducing actin polymerization. Hence, we have devel-oped a novel cell line mimicking signaling in CML PMNL which could serve as a preclinical model for CML. Studies on actin polymerization pathway in this cell line have identified rhoA as the therapeutic target for CML.

2. Materials and methods

2.1. Reagents

Antibodies, probes and kits were obtained from various sources listed here: anti-abl, anti-actin, anti-rac1, anti-rhoA and alkaline phosphatase conjugated goat anti-rat antibody (anti-rat-AP) [Santacruz Inc., USA]; anti-H-ras [Oncogene, USA]; enhanced

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chemiluminescence kit containing alkaline phosphatase conjugated goat anti-rabbit antibody (GAR-AP) [New England Biolabs Inc., USA]; Bodipy conjugated goat anti-rabbit antibody (Bodipy GAR), Alexa 488-conjugated goat anti-mouse antibody (GAM), Fluorescein labelled *n*-formyl-Norleucyl-Leucyl-Phenyl alanyl-Norleucyl-Tyrosyl-Lysine (fNLPNTL-FL) [Molecular Probes, USA]; FITC conjugated anti-ras and TRITC conjugated anti-rac1, FITC/PE conjugated monoclonal antibodies for CD13, CD14, CD15, CD33, CD3, CD7 and CD19 [Becton-Dickinson, USA]; *n*-formyl-Methionyl-Leucyl-Phenyl alanine (fMLP), goat anti-mouse antibody alkaline phosphatase conjugated (GAM-AP) [Sigma, USA], cell counting kit-8 (CCK-8) [Dojindo Laboratories, Japan], GTPase biochem kits for active forms [Cytoskeleton Inc., USA] Superscript First-Strand synthesis system for RT-PCR [Gibco BRL Life technologies, USA] and Sequenase version 2.0 PCR product sequencing kit [Amersham Life Science, USA].

2.2. Establishment of a cell line

2.2.1. Cell culture and transfection

To 2 million RIN9815 cells [4] suspended in 200 μL serum deficient medium, plasmids pGD210 that contains bcr-abl gene and pUT531 that codes for phleomycin resistance (10 μg of each) were added and subjected to a 280-Volt pulse. After 48h, phleomycin (20 μg/mL) resistant clones were selected and screened for bcr-abl expression. RIN9815 and its transfected clones were grown in RPMI 1640 containing 2 g/L of sodium pyruvate, 1 × AB-AM and 10% (v/v) fetal bovine serum, at 37 °C in a humidified atmosphere containing 5% CO_2. For K562 [5] and BaF3/bcr-abl/T315I cells, RPMI without sodium pyruvate was used.

2.2.2. Expression of bcr-abl

2.2.2.1. Immunofluorescent staining. Cells fixed with paraformaldehyde and permeabilized with Triton-X 100 were incubated at 37 °C with anti-abl antibody (1:100), followed by Bodipy GAR (1:750), each for 45 min. The cells were analyzed by flow cytometry using FACSCalibur (Becton-Dickinson, USA).

2.2.2.2. Western blotting. Cells (5×10^5) were lysed in RIPA buffer containing 40 × Complete™ (protease inhibitors cocktail, Roche, Germany) and phosphatase inhibitors. Western blot analyses were performed using anti-abl antibody (1:500) and enhanced chemiluminescence kit [3]. K562 cells were used as a positive control.

2.2.2.3. RT-PCR. RNA isolated by the Trizol method [6] was transcribed with Superscript First-Strand synthesis system for RT-PCR. PCR was performed for bcr-abl [7] and G3PDH expression.

2.2.2.4. Sequencing. The PCR products for bcr-abl were sequenced using Sequenase version 2.0 PCR product sequencing kit [8].

Of the various clones tested, clone b1 consistently showed considerable levels of bcr-abl expression. Therefore for further studies, clone b1 was characterized and it is referred as RIN9815/bcr-abl. The parental cell line RIN9815 was used as a control to understand the effect of bcr-abl transfection.

2.3. Characterization of RIN9815/bcr-abl

2.3.1. Morphology

Cells were observed under Nikon microscope DIAPHOT-TMD (objective 10 ×/0.25 NA) and photographed using Nikon F-601M camera.

2.3.2. Growth curve and cell cycle

Cells seeded at a density of 10^5 cells per mL, were harvested at 0, 24, 48, 72 and 96 h, counted and then fixed and stained with propidium iodide [9]. Cell cycle analysis was done by using flow cytometer.

2.3.3. Cytogenetics

Cells cultured for 24 h were treated with cholchicine (0.2 μg/mL) for 90 min. Metaphase spreads were prepared [10] and imaged using using Zeiss MC80DX camera attached to Axioskop 2 MOT microscope (objective 100 ×/1.4 NA). Metaphases were analyzed using Metasis software.

2.3.4. Cellular markers

Expression of epithelial markers – keratins was studied by western blotting as mentioned above, using anti-pan cytokeratin antibody (1:1000) and GAM-AP (1:50,000). FBM and RINm5F cells were used as positive controls. Immunophenotyping was done by FACSCalibur, using fluorescent antibodies for myeloid markers – CD13, CD14, CD15 and CD33, and lymphoid markers – CD3, CD7 and CD19.

2.3.5. Tumourigenicity

One million cells were subcutaneously injected into 5–6 weeks old nude mice (NIH-III). The mice were sacrificed when the tumour diameter reached about 1 cm. Part of the tumour and various organs were fixed in 10% formalin. Histology was done using haematoxylin and eosin. Images were captured using Zeiss Axiovert 200 microscope (objective 63 ×/0.80) attached to Sony DXC-390P camera, using PALM® Robo software. A small piece of the tumor was used to study bcr-abl expression and part of it was frozen in liquid N2. About 30 mg of tumour from nude mouse was injected in another nude mouse to study tumour development.

2.4. Comparison with CML PMNL

2.4.1. Expression of surface FPR (S-FPR) and total FPR (T-FPR)

To estimate FPR, paraformaldehyde fixed, non-permeabilized and permeabilized cells were stained with anti-FPR antibody. In non-permeabilized cells, the antibody would access only S-FPR that is present on the surface of the cell. To estimate the T-FPR pool, i.e. the FPR expressed on cell surface along with intracytoplasmic pool, paraformaldehyde fixed cells were stained after permeabilization with Triton-X 100. Permeabilization would enable the antibody to access FPR present on cell surface and in various intracytoplasmic compartments, giving estimate of T-FPR. For staining, cells were incubated with equal volume of 1:5 diluted FITC labelled anti-*N*-terminal FPR antibody (raised in-house), at 37 °C for 30 min. The cells were analysed flow cytometrically.

2.4.2. FPR–ligand binding

An amount of 100 nM fNLPNTL-FL was added to 10^7 cells. After incubation for 30 min at 4 °C or 37 °C, the cells were fixed with 4% paraformaldehyde [11] and analyzed by flow cytometry.

2.4.3. FPR trafficking

Cells grown on polylysine (0.1%) coated coverslips for 48 h were incubated in serum free RPMI 1640 containing 1% BSA and cycloheximide (100 μg/mL), for 3 h at 37 °C. To study trafficking of T-FPR, cells were stimulated with 400 nM fMLP for 0.5, 1, 2.5, 5, 10, 20, 30, 45 and 60 min at 37 °C. Cells were then fixed, permeabilized and stained using anti-*C*-terminus FPR antibody (1:50) and Bodipy GAR (1:500). To study the fate of fNLPNTL-FL bound FPR, the cells incubated with 400 nM of fNLPNTL-FL as described above, were fixed. To precisely study localization of the molecules, Z-series images of 1 μm thickness were analysed by BioRad MRC 1024 laser scanning confocal microscope (LCM).