Expression and effects of inhibition of type I insulin-like growth factor receptor tyrosine kinase in mantle cell lymphoma

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Online Supplementary Methods

Cell lines

The P6 (mouse BALB/c3T3 fibroblasts over-expressing human IGF-IR) and R- (mouse 3T3-like fibroblasts with targeted ablation of *Igf1r*) cell lines (generous gifts from Dr. Renato Baserga, Philadelphia, PA, USA) were used as positive and negative controls, respectively, for the expression of IGF-IR. In some experiments, the ALK$^+$ T-cell lymphoma cell line Karpas 299 was also used as a positive control for the expression of IGF-IR. The chronic myelogenous leukemia cell line K562 and the spontaneously immortalized Schwann cells RSC96 (ATCC, Manassas, VA; USA) were used as positive and negative controls, respectively, for the expression of IGF-I. Human peripheral blood CD19$^+$ B-lymphocytes were purchased from STEMCELL Technologies (catalogue number: PB010-P-F; Vancouver, BC, Canada). B-lymphocytes and MCL cell lines were cultured in RPMI 1640 medium (HyClone, South Logan, UT, USA) supplemented with 15% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). Alternatively, RPMI 1640 (Karpas 299, K562) or DMEM (P6, R$^-$, RSC96) supplemented with 10% FBS was used for the specified cell lines. In order to assess whether IGF-I, which is present in FBS, might contribute to the expression and/or activation of IGF-IR, MCL cell lines were cultured in FBS-deprived medium for 24 h, and then quantitative real-time polymerase chain reaction (qPCR) and western blot analyses were performed.

Antibodies

Antibodies obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) included pIGF-IR (Tyr1131; sc-101703), IRS-1 (sc-7200), pIRS-1 (Tyr941; sc-17199-R), cyclin B1 (sc-7393), cyclin D1 (sc-8396), Cdc2 (sc-52316), pCdc2 (Thr14/Tyr15; sc-12340-R), and pTyr (sc-7020); antibodies from Cell Signaling Technology (Danvers, MA, USA) were pIGF-IR (Tyr1131; 3021), total caspase-3 (9668), cleaved caspase-3 (9661), caspase-8 (9746), caspase-9 (9502), Akt (9272), pAkt (Ser473; 587F11), Jnk (9252), and pJnk (9255); IGF-IR (39-6700) was from Zymed Laboratories (South San Francisco, CA, USA); β-actin (A-2228) was from Sigma (St. Louis, MO, USA); and total PARP (556362) and cleaved PARP (552596) were from BD Biosciences (San Jose, CA, USA).

Reverse transcriptase polymerase chain reaction and quantitative real-time polymerase chain reaction

Total RNA was isolated and purified using the RNAeasy Mini Kit (Qiagen, Valencia, CA, USA). Optical density readings were obtained using DNA/Protein Analyzer (Beckman Coulter, Fullerton, CA, USA). Reverse transcription was performed using ThermoScript RT (Invitrogen, Carlsbad, CA, USA). PCR reactions were performed using a HotStar Master Mix kit (Qiagen). Amplification was performed in a PTC100 thermal cycler (MJ Research). The sequences of the IGF-IR and IGF-I primers have been detailed previously.[1,2] β-actin (RDP-38; R&D Systems) was used as an internal control. PCR products were detected by ethidium bromide staining on a 1.5% agarose gel and visualized using a FluorChem 8800 imaging system (Alpha Innotech, Santa Clara, CA, USA). Quantitative real-time PCR was also used to measure IGF-I mRNA levels. The primer/probe sets were from Applied Biosystems and included 18S ribosomal RNA (4319413E-0402011) and IGF-I (Hs01547657_m1).

Measurement of IGF-IR antigen density per cell

The relative IGF-IR antigen density per cell was assessed using flow cytometry. Phycoerythrin (PE)-labeled anti-human IGF-IRα antibody (555999; BD Biosciences) or PE-labeled anti-mouse IgG_1 isotype control (555749; BD Biosciences) was used. IGF-IR antigen density per cell was determined according to the manufacturer's instructions by using the QuantiBRITE PE kit (340495; BD Biosciences) and was calculated considering that the PE:monoclonal antibody ratio was 1:1.

Western blotting and immunoprecipitation

The lysis buffer used in our experiments contained 25 mM HEPES (pH 7.7), 400 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 0.5% Triton X-100, 0.1 mM PMSF, 3 mM DTT, a phosphatase inhibitor cocktail (20 mM β-GP, 1 mM Na_3VO_4; Roche, Mannheim, Germany), and a protease inhibitor cocktail (10 μg/mL leupeptine, 2 μg/mL pepstatin, 50 μg/mL antipain, 1× benzamidine, 2 μg/mL aprotinin, 20 g/mL chymostatin; Roche). For immunoprecipitation, the anti-IGF-IRβ antibody (Zymed) was added to the cell lysate and incubated overnight at 4°C. Agarose beads conjugated with A/G were then added and incubated for 4 h at 4°C. The immunocomplexes were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). For western blotting, proteins (50-80 g) were electrophoresed on 6% to 12% SDS-PAGE. The proteins were transferred to nitrocellulose membranes and probed with specific primary antibodies and then with the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz; and GE Healthcare, Cardiff, UK). Proteins were detected using a chemiluminescence-based kit (GE Healthcare).

Immunohistochemical staining

Slides were incubated overnight at 4°C with anti-IGF-IR antibody (1:30; Zymed) or anti-pIGF-IR (1:10; Santa Cruz). Slides were washed three times for 10 min and incubated with secondary antibody LINK and then with secondary antibody streptavidin each for 30 min. Photomicrographs were captured using a Nikon Microphot FXA microscope (Nikon Instruments, Melville, NY, USA) and an Olympus DP70 camera (Olympus America, Melville, NY, USA).

Exclusion of staining with trypan blue dye

Cell viability was analyzed using exclusion of staining with trypan blue dye. Briefly, equal volumes of cell suspensions and trypan blue dye (Cambrex Biosciences, Walkersville, MD, USA) were mixed. Stained cells were considered dead and were counted using light microscopy and a hemocytometer.

Apoptosis and cell cycle analysis

Apoptosis was evaluated using flow cytometric analysis after staining the cells with annexin V-FITC and propidium iodide (PI; BD Pharmingen). Cytochrome c release was performed by using a commercially available kit (ab65311; AbCam, Cambridge, MA, USA) according to the manufacturer's protocol. Analysis of the cell cycle was performed using a commercially available kit (CycleTEST PLUS DNA Reagent Kit, BD Biosciences), whereby PI-stained nuclei were analyzed by flow cytometry (FACscan, BD Biosciences). The phases of the cell cycle were analyzed by ModFit LT 2.0 software (Verity Software House, Topsham, ME, USA). Morphological features of apoptosis and cell cycle arrest were studied by light microscopic examination of cytospin slides stained with Giemsa.

Tyrosine kinase activity

IGF-IR tyrosine kinase activity was measured using an Universal Tyrosine Kinase Assay Kit (Takara Bio, Pittsburg, PA, USA) according to the manufacturer's protocol. The optical density readings were measured at a 450 nm wavelength using an enzyme-linked immunosorbent assay (ELISA) plate reader.

MTS assay

Cell proliferation was evaluated using the One Solution Cell Proliferation Assay (MTS) kit (Promega, Madison, WI, USA). Cells were seeded in 96-well plates at a concentration of 10,000 cells/well. The MTS reagent was added and then incubated at 37°C in 5% CO_2 in humidified air for approximately 4 h. Optical density measurements were obtained using an ELISA plate reader.

Statistical analysis

Student's t-test for paired data or analysis of variance (ANOVA) for repeated measures was used when appropriate. Dunnett's method was used to adjust for type I errors.

References

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2. Shi P, Chandra J, Sun X, Gergely M, Cortes JE, Garcia-Manero G, et al. Inhibition of IGF-IR tyrosine kinase induces apoptosis and cell cycle arrest in imatinib-resistant chronic myeloid leukemia cells. J Cell Mol Med. 2010;14(6B):1777-92.