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Novel human multiple myeloma cell line UHKT-893

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ABSTRACT

We established and characterized a new IL-6 dependent multiple myeloma (MM) cell line UHKT-893 from the bone marrow of a relapsed 57-year-old woman.
Results: Using nephelometry, cells with plasma cell phenotype and morphology were found to secrete IgG and free kappa (κ)-light chain of immunoglobulin. κ-Light chain was also recognized intracellularly by flow cytometry and by mass spectrometry. VH4-39 region of IgVH genes was rearranged and somatically hypermutated. Cytogenetic analysis of cells revealed new chromosome abnormalities in all breakpoints unique in both MM patients and cell lines – t(1;6), t(1;11), t(5;15), t(5;21), +der(11;15) and der(16). IL-6 independent subline UHKT-893a was established by adaptation to descending IL-6 concentration, while the original cell line keeps on maintaining its IL-6 dependency.
Conclusion: The cell line provides a suitable material for cellular and molecular studies of tumor abnormalities, with potentially unique mutagenic features of myeloma disease. It may be utilized for human hybridoma construction and vaccine development. Both IL-6 dependent and independent cell clones represent an important model for studies of myeloma cell growth and resistance emerging during targeted therapy.

1. Introduction

Multiple myeloma (MM) is a hematopoietic neoplasia of terminally differentiated B-cells into plasma cells. For the study of etiopathogenesis and for development of effective treatment of MM, a bulk of neoplastic cells is required exceeding the limited and temporary cell collections from patients. Permanent myeloma cell lines established from patients provide easily accessible and indispensable model material. Establishment of permanent cell lines from myelomas and other hematological neoplasias is still a very difficult and rare event, and is rather a matter of coincidence because factors critical for the growth and survival of myeloma stem cells *in vitro* are still poorly understood. A higher success rate in the establishment of permanent malignant hematopoietic cell lines has been achieved in patients at relapse, in terminal or refractory phase rather than at diagnosis, during therapy or at blast crisis [1].

Interleukin 6 (IL-6) is a major growth and survival factor for myeloma cells *in vivo*, primary myeloma cells *in vitro* and also for myeloma cell lines [2,3]. Similarly, IL-6 acts on non-malignant human plasmablasts [4]. Several studies demonstrated or suggested that IL-6 may act by autocrine, intracrine and paracrine manner [2,5]. We could not rule out that IL-6 acts as an intracrine factor [6]. Up to date, 25% of myeloma and 47% of plasma cell leukemia cell lines have been established as IL-6 dependent [1]. During long-term cultivation, cells may become

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independent of growth and survival factors, as was documented on HL461 myeloma cell line originally dependent on stromal-cell-conditioned medium [7].

In the present study we established and characterized a new human IL-6 dependent myeloma cell line UHKT-893 and selected its IL-6 independent subclone UHKT-893a that can be utilized in both the research of multiple myeloma and other technological and therapeutic approaches.

2. Materials and methods

2.1. Patient

57-Year-old woman suffered from a backache since July 2007 and was diagnosed with multiple myeloma, clone IgG-K, stage I of International Staging System, stage IIIA according to Durie–Salmon staging system, on February 2008. The level of monoclonal immunoglobulin (mIg) was 46.5 g/L. According to cytology and FACS analysis findings, 62% and 45% myeloma plasma cells was present in the bone marrow, respectively. Generalized skeletal disruption – tiny cranial and long bone focuses, several compressive fractures of vertebra on the transition between the thoracic and lumbar backbone – occurred. The patient was a chronic smoker and suffered from arterial hypertension from the spring 2008.

2.2. Patient therapy

Briefly, during the year of diagnosis the patient was treated by 4 cycles of Velcade–Thalidomid–Dexona chemotherapy, leading to a partial remission (18.4 g/L mIg, 8.4% and 0.4% plasma cells, as noted above). Subsequently, the patient received mobilization chemotherapy (cyclophosphamide, 2.5 g/m^2) and G-CSF (neupogen, 10 μg/kg/daily), leading to a successful separation of peripheral blood progenitor cells for autologous stem cell transplantation. This was conditioned by a single application of myeloablative dose of melphalan (200 mg/m^2) resulting in a partial response. Relapse on February 2010 necessitated a second line therapy. However, before the therapeutic application, a rapid progression of cardiac insufficiency accompanied by bronchopneumonia led to the patient's death one month later.

2.3. Cell line establishment and cultivation

Bone marrow sample of the patient in relapse was obtained on 16th February 2010 with the patient's informed consent. Erythrocytes and granulocytes were removed from anticoagulated sample by separation on Histopaque®-1077 (Sigma–Aldrich) and remaining mononuclear cells in high density (approx. 1×10^6 cells/mL) were cultivated in RPMI 1640 medium with 10% FBS (both from Biochrom AG), 20 ng/mL G-CSF (Filgrastim, Roche) and 100 U/mL penicillin/100 μg/mL streptomycin (Sigma) in 5% CO_2 atmosphere at 37 °C. Whenever were the cells separated from patient's autologous feeder stromal layer and transferred into a new flask, the cells required supplementation of exogenous recombinant human IL-6 (Invitrogen) at a concentration of 1 ng/mL instead of G-CSF for further growth and division.

2.4. Growth characteristics

2.4.1. Morphology

Cytospin preparations were routinely stained by Giemsa in the cytomorphological laboratory to regularly assess the cell type morphology and the presence of mitoses. The presence of senescent cells was assessed by β-galactosidase staining (Cell Signaling). The amount of senescent cells was estimated several times to check the state of the continuously growing cell line in culture.

2.4.2. Splitting

Cell cultures were passaged in a 1:3 ratio twice or once a week.

2.4.3. Doubling time

It was analyzed from cell counts during their growth phase by GraphPad Prism version 5.02 software.

2.4.4. Measurement of proliferation

Cell growth has been regularly monitored in around 10 mL of medium and numbers of living cells counted in Bürker chamber or on automated cell counter (TC10, BioRad) were normalized to this volume for comparability. Optimal IL-6 concentration stimulating the cell growth was determined based on the cell proliferation checked microscopically and using colorimetric assay employing WST-1 reagent (Roche). For colorimetric assay, cells were incubated for 3 h with WST-1 reagent and absorbance of the samples was measured at $A_{450\,nm}$ with reference wavelength $A_{690\,nm}$ according to the manufacturer's protocol.

2.4.5. Measurement of viability

Cells were stained with trypan blue (Sigma) and viable and dead cells were counted in Bürker chamber or cell counter. The values are given in %.

Concurrent attempts to obtain IL-6 independent cell line have been performed by gradual decreasing of IL-6 concentration, or by alternating the culture with and without IL-6 with the aid of spontaneous adherence of live cells to a layer of poly-L-lysine (Sigma) in order to easily remove the harmful bulk of floating dead cells.

2.4.6. Classical cytogenetic analysis

For the cytogenetic analysis, bone marrow cells were cultured for 24 h in RPMI 1640 medium with 10% fetal calf serum without stimulation. Chromosomal preparations of the patient's bone marrow and UHKT-893 cells were made according to the standard techniques using colcemide, hypotonic treatment, fixation in methanol/acetic acid and G-banding with Wright stain. For karyotyping, mitoses were analyzed using IKAROS (MetaSystems) or LUCIA (Laboratory Imaging) imaging systems.

2.4.7. FISH analysis

Interphase fluorescent *in situ* hybridization (FISH) with cytoplasmic immunoglobulin light chain staining (cIg FISH) was performed on bone marrow cells. Commercial DNA probes (Abbott Molecular) were used for detection of deletions del(13)(q14) (RB1 gene)/monosomy of chromosome 13, del(17)(p13) (TP53 gene) and translocations involving IgH gene (14q32). For 1q21 gain status, cIg FISH with ON MM 1q21/SRD (1p36) DNA specific probe from Poseidon (Kreatech Diagnostics) was established.

2.4.8. mFISH analysis

Multicolor FISH (mFISH) was performed using SpectraVision (Abbott) on 18-month-old UHKT-893 cells. Image capturing and acquisition was processed using Nikon Eclipse 90i fluorescence microscope and LUCIA imaging system for FISH.

2.4.9. Immunophenotypic analysis by flow cytometry

Live cells were tested for the expression of molecules by antibodies to surface antigens CD1a, CD19, CD20, CD21, CD25 (Beckman Coulter Inc.); CD3, CD28, CD33, CD38, CD45, CD56, CD117, CD138 (Invitrogen); CD4, CD13, CD27 (eBioscience); CD5 (L17F12, gift from Prof. Ronald Levy, Stanford, CA); CD8 (BioLegend); CD9 (Hybritech); CD10, CD24 (Ortho Diagnostic System Inc.); CD15 (Becton Dickinson); CD34, CD44 (Exbio); CDw65, CD89, CD95, CD98 (Immunotech); CD96 (Abnova Corporation); CD105 (Ancell); CD184 (Diaclone); HLA-DR (Orthomune); cytoplasmic κ and λ light immunoglobulin (Ig) chains (Dako). Cells incubated with unlabeled primary antibodies were stained by RPE-conjugated F(ab′)2 fragment of goat or rabbit anti-mouse immunoglobulins (DAKO). Washing and incubation steps were carried out with PBS. Other cell lines with known antigenic expression were used as negative and positive controls – CML-T1, JURL-MK1, KARPAS-299 (DSMZ); JURKAT (ECACC), HEL (from Dr. Paul Martin, Fred Hutchinson Cancer Research Centre, Seattle, WA, USA), HL-60 (from Dr. R.C. Gallo, National Cancer Institute, Bethesda, USA), MOLM-7 (from Dr. Y. Matsuo, Fujisaki Cell Center, Hayashibara Biochemical Laboratories, Inc., Okayama, Japan); KG-1 and NALM-16 (from Dr. Jun Minowada, Roswell Park Memorial Institute, Buffalo, NY, USA). Intensity of fluorescence was measured by flow cytometry (FACS – Coulter Epics XL). Only the population of live cells was gated and evaluated. The used secondary antibody did not provide any nonspecific positivity.

2.4.10. Immunoglobulin secretion

Secretion of immunoglobulins and their free light chains from cells to medium was routinely measured by a nephelometric technique (Institute of Clinical Biochemistry and Laboratory Diagnostics, General University Hospital, 1st Medical Faculty, Charles University, Prague).

2.4.11. Mass spectrometry

2D electrophoresis was performed according to a described procedure [8]. MALDI-TOF analysis was employed to analyze protein spots randomly selected originally for the purpose of method tutorial for students. Protein spots from 2D electrophoresis gel of whole cell-lysate after standard in-gel digestion were analyzed in the Laboratory of Mass Spectrometry (Faculty of Science, Charles University, Prague). Resulting protein mass spectra were compared with Genbank non-redundant protein database by Mascot software. Proteins with significant score ($p < 0.001$) were identified and confirmed by tandem mass spectrometry.

2.4.12. Sequence analysis of IgVH gene rearrangement

RNA was isolated using TriZol reagent according to the manufacturer's instructions (Invitrogen). cDNA was synthesized using SuperScript II and random hexamers (Invitrogen). Seven IgVH (immunoglobulin heavy chain variable region) families were amplified in six individual PCRs (VH1 and VH7 families in the same reaction mixture) employing primers described by Pekova et al. [9] using Ampli Taq Gold Polymerase (Applied Biosystems) and "touch-down" PCR program. The temperature of annealing steps was gradually descended from 65 °C to 50 °C. PCR products were separated on 2% agarose with ethidium bromide. The bands around 300 bps were cut out and purified with QIAquick Gel Extraction kit (Qiagen). Sequencing was performed on ABI PRISM 3500 Genetic Analyzer using Big Dye Terminator 3.1 kit (Applied Biosystems). The resulting sequences were analyzed in Chromas 2.31 program and aligned to the nearest IgVH germLine sequences using databases of