ELSEVIER

European Journal of Cancer 41 (2005) 178–187

European Journal of Cancer

www.ejconline.com

A proteomic approach to tumour target identification using phage display, affinity purification and mass spectrometry

Cecilia A.W. Geuijen, Nora Bijl, Renate C.M. Smit, Freek Cox, Mark Throsby, Therèse J. Visser, Mandy A.C. Jongeneelen, Alexander B.H. Bakker, Ada M. Kruisbeek, Jaap Goudsmit, John de Kruif *

Crucell Holland B.V., P.O. Box 2048, 2301 CA Leiden, The Netherlands

Received 3 June 2004; received in revised form 24 September 2004; accepted 7 October 2004
Available online 11 November 2004

Abstract

Tumour-associated cell surface markers are potential targets for antibody-based therapies. We have obtained a panel of myeloid cell binding single chain variable fragments (scFv) by applying phage display selection on myeloid cell lines followed by a selection round on freshly isolated acute myeloid leukaemia (AML) blasts using flow cytometry. To identify the target antigens, the scFv were recloned and expressed in an IgG_1 format and tested for their ability to immunoprecipitate cell surface proteins. The IgGs that reacted with distinct cell membrane extractable proteins were used in large-scale affinity purification of the target antigen followed by mass-spectrometry-based identification. Well-characterised cell surface antigens, such as leukocyte antigen-related receptor protein tyrosine phosphatase (LAR PTP) and activated leukocyte adhesion molecule (ALCAM) in addition to several unknown proteins, like ATAD3A, were identified. These experiments demonstrate that phage antibody selection in combination with affinity chromatography and mass spectrometry can be exploited successfully to identify novel antibody target molecules on malignant cells.

Keywords: Phage display; Tumour antigen; Single chain Fv; AML; Myeloid; LAR PTP; ALCAM; ATAD3A

1. Introduction

The treatment of cancer by antibody therapy is rapidly becoming established in clinical practice. Currently, there are eight Food and Drug Administration (FDA) approved monoclonal antibodies for oncology applications, while an additional seven are in late-stage clinical development [1]. However, application of general antibody therapy is hampered by the lack of tumour-associated markers. Therefore, the identification of novel targets is a crucial step in designing and developing antibody-based therapies.

Antibody phage display is one of the technologies that can lead to the identification of novel target molecules. Several research groups have used antibody phage display selection strategies to isolate antibodies that bind to surface markers on tumour cells. This has resulted in phage antibodies directed against human melanoma cells [2–5], human lung carcinoma [6] and colorectal carcinoma cells [7,8]. However, the transition from the selection of cell-specific phage antibodies to the identification of the cellular target is still a major hurdle. For the identification of antigens, a genomic approach using cDNA expression cloning and a proteomic approach using affinity chromatography and mass spectrometry can be employed.

In this study, phage antibody selections were performed on myeloid cells resulting in the isolation of

* Corresponding author. Tel.: +31 71 5248779; fax: +31 71 5248702.
E-mail address: j.dekruif@crucell.com (J. de Kruif).

0959-8049/$ - see front matter
doi:10.1016/j.ejca.2004.10.008

a panel of myeloid cell binding antibodies. To identify the target antigens, affinity purification followed by one-dimensional electrophoresis and mass spectrometry (MS)-analysis was used. The resulting antigen panel consists of several well-characterised proteins, leucocyte antigen-related receptor protein tyrosine phosphatase (LAR PTP), activated leucocyte adhesion molecule (ALCAM) and nicotinamide nucleotide adenylyltransferase, in addition to several hypothetical proteins. Restrictive expression of a selection of these target antigens on acute myeloid leukaemia (AML) was confirmed by fluorescence activated cell sorting (FACS) analysis with phage antibodies. Together, these results demonstrate the power of phage display in combination with affinity purification and MS for the identification of novel cell type-specific cell surface receptors.

2. Materials and methods

2.1. Cells and antibodies

All cell lines used were derived from the American Type Culture Collection (ATCC). NB4, HL60 and K562 were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% heat inactivated foetal bovine serum (FBS-HI) and 2 mM L-glutamine. HEK293T was cultured in Dulbeccos modified eagle medium (DMEM) supplemented with 10% FBS-HI and 0.4 mM L-glutamine. HEp-2 was cultured in DMEM supplemented with 10% FBS-HI and 2 mM L-glutamine. The colon adeno carcinoma cell line LS174T was cultured in DMEM supplemented with 10% FBS-HI.

Peripheral blood buffy coats were obtained from the Sanquin Blood Supply Foundation. Leukaemia cells were obtained from peripheral blood of newly diagnosed AML patients after informed consent and classified according to the French–American–British (FAB) classification.

Monoclonal antibodies used in this study: CD16-fluorescein isothiocyanate (FITC), CD19-APC, CD32-FITC, and CD33-phycoerythrin (PE) were obtained from Pharmingen, CD14-FITC, CD3-FITC, CD33-APC, CD34-PE and CD45-PerCP were from Becton–Dickinson and monoclonal anti-myc antibody (9E10) was from Santa Cruz.

2.2. Flow cytometry using scFv-phage

Reactivity profiles of phage antibodies were analysed using FACS analysis; essentially as described before in [9], except that biotinylated anti-M13 mAb (Roche) followed by streptavidin-PE (Caltag) was used to detect the cell bound phages.

2.3. Phage selections

Phage antibodies were selected using a semi-synthetic antibody phage display library [9] rescued with the CT helper phage [10]. An aliquot of the phage library ($\sim 1.5 \times 10^{13}$ colony forming units (cfu)) was blocked for 30 min on ice in RPMI 1640 containing 10% FCS (RPMI/FCS). Subsequently, the library was subtracted three times with 2.3×10^8 peripheral blood cells (PBL) in 10 ml RPMI/FCS for 2 h at 4 °C, to deplete phage antibodies binding antigens on non-tumorigenic cells. After centrifugation to remove the absorber cells, the phage-containing supernatant was incubated in parallel with 4×10^6 cells of HL60, K562 and NB4 for 2.5 h at 4 °C. After removal of unbound phages, cell bound phages were eluted and rescued as described in [9,10]. A second selection round on primary AML tumour cells (CD34+ blasts, FAB M0) was performed by incubating blocked phages for 2.5 h at 4 °C with 5×10^6 CD34PE labelled AML cells. After washing in RPMI/FCS, CD34+ cells with attached phages were sorted on a FACSVantage flow cytometer [9]. Attached phages were eluted, rescued and monoclonal phage preparations were generated for binding analysis on PBL, cell lines and AML tumour samples as described previously in [10]. Binding clones were sequenced to identity unique clones as described previously in [11].

2.4. Generation of human IgG_1 antibodies

The engineering and production of the human IgG_1 monoclonal antibodies was performed as described in detail by Boel and colleagues in [12]. The variable regions of the scFv were recloned in separate vectors for IgG_1 heavy and light chain expression. VH and VL regions from each scFv were polymerase chain reaction (PCR) amplified using primers to append restriction sites and restore complete human frameworks. IgG_1 were produced as described previously in [12] and purified on protein-A columns followed by buffer exchanging in PBS over size-exclusion columns (Amersham Biosciences).

2.5. Biotinylation of cell surface molecules and immunoprecipitation

Small-scale immunoprecipitation procedures were performed of each antibody/antigen combination to determine the optimal conditions in large-scale affinity purification.The cell surfaces of 10^8 human LS174T, HEp-2 and NB4 were biotinylated for 1 h at room temperature (RT) with 2 mg Sulfo-NHS-LC-LC-biotin per 10^8 cells (Pierce) in physiological buffer (0.2 M phosphate buffer containing 0.12 M NaCl, pH 7.4 (PBN). After blocking of free biotin with 10 mM glycine (Gibco) in PBN, the cells were lysed at a concentration