Acta Histochemica 114 (2012) 594–602

Contents lists available at SciVerse ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

MDR-1-overexpression in HT 29 colon cancer cells grown in SCID mice

Udo Schumacher[a,*], Nina Nehmann[a], Elizabeth Adam[b], Dhia Mukthar[b], Itzchak N. Slotki[c], Hans-Peter Horny[d], Marcel J. Flens[e], Brigitte Schlegelberger[f], Doris Steinemann[f]

[a] Department of Anatomy and Experimental Morphology and University Cancer Center, University Medical Center Hamburg-Eppendorf, Martinistraße 52, D-20246 Hamburg, Germany
[b] Infection, Inflammation and Repair Division, University of Southampton, Southampton General Hospital, Tremona Road, Southampton, Hampshire SO16 6YD, United Kingdom
[c] Department of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91904, Israel
[d] Institute for Pathology, Ansbach Hospital, Escherichstraße 6, 91522 Ansbach, Germany
[e] Institute for Pathology, Academic Hospital, Free University, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands
[f] Institute of Cell and Molecular Pathology, Hannover Medical School, Carl Neuberg-Str.1, 30625 Hannover, Germany

ARTICLE INFO

Article history:
Received 23 October 2011
Received in revised form 7 November 2011
Accepted 8 November 2011

This work is dedicated to Prof. Thomas Heinzeller, Anatomische Anstalt München, on the occasion of his retirement.

Keywords:
Gene expression
HT29 colon cancer cells
MDR1-overexpression
Metastasis
Multidrug-resistance
SCID mouse
Regulation

ABSTRACT

The multidrug-resistance 1 (MDR-1) P-glycoprotein (Pgp) is a transmembrane transporter system, which actively pumps cytotoxic drugs out of the cell. MDR-1 acquired *in vitro* differs from MDR-1 acquired *in vivo*, but has important consequences on the cellular phenotype and metastatic behavior. Here we report that the human colonic cancer cell line HT29 (MDR-1 negative) is more malignant than its MDR-1 overexpressing variant (HT29 MDR-1 positive). HT29 MDR-1 negative cells produce undifferentiated signet ring carcinomas when implanted subcutaneously into SCID mice, while HT29 MDR-1 positive cells form tumors with tubular structures, but without signet ring cells. Immunohistochemical proliferation marker analysis revealed that the MDR-1 positive cells proliferate much more slowly than the MDR-1 negative cells. MDR-1 overexpression results in a less differentiated phenotype at the cellular level (absence of mucin producing cells) but in a more differentiated phenotype at the tissue level (tubule formation). In addition, lectin binding patterns including that of *Helix pomatia* agglutinin (HPA), an indicator of metastatic potential, differed between the two cell lines. HT29 MDR-1 positive cells had less HPA binding sites than HT29 MDR-1 negative counterparts and metastasized less frequently in SCID mice. As slow proliferation, low degree of differentiation and multidrug-resistance is a hallmark of cancer stem cells and all were present in MDR-1 positive tumors, it is attractive to speculate that they represent a stem cell rich tumor. As shown by global gene expression analyses, genes involved, e.g. in cell adhesion, glycosylation and signal transduction, were deregulated in MDR-1 positive tumors compared to MDR-negative tumors. Overexpression of E-cadherin and carcinoembryonic antigen-related cell adhesion molecules 1 (CEACAM1) may provide clues to the mechanisms responsible for the reduced metastatic potential of MDR-1 overexpressing tumors. Since drug treatment shifted the cells towards a less metastatic phenotype in this *in vivo* model, it seems conceivable to achieve this using drug treatment also in a clinical situation.

Introduction

The mammalian multidrug-resistance 1 (MDR-1) phosphoglycoprotein (Pgp) is a 170 kDa transmembrane glycoprotein, which actively eliminates a wide range of chemotherapeutic drugs in those malignant cells overexpressing it, causing considerable problems in the treatment of metastatic neoplastic disease (Gottesman and Pastan, 1993). We therefore wanted to investigate growth and differentiation behavior in association with MDR-1 overexpression in a human cancer cell line/severe combined immunodeficient (SCID) xenograft mouse system. The human colon cancer cell line, HT29, implanted into SCID mice was chosen as the model system for several reasons. The SCID mouse harboring human tumors is superior in the assessment of cell growth and differentiation to *in vitro* culture systems, since it has been shown that certain differentiation steps occur only *in vivo* and not *in vitro* (Schumacher and Mohamed, 1996). In addition, tumor cell heterogeneity in the SCID mouse resembles that occurring in patients, which is not the case *in vitro* (Schumacher and Mohamed, 1996). When HT29 cells were subcutaneously transplanted into SCID mice, spontaneous distant metastases formed and it is the prevention of metastatic disease which is the main rationale for using chemotherapy. This HT29 SCID mouse xenograft model of metastatic colon cancer is of particular interest since

* Corresponding author at: Institute for Anatomy II: Experimental Morphology, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany.
E-mail address: uschumac@uke.uni-hamburg.de (U. Schumacher).

0065-1281/$ – see front matter © 2011 Elsevier GmbH. All rights reserved.
doi:10.1016/j.acthis.2011.11.004

it shows a close correlation to the clinical situation (Schumacher and Adam, 1997). The binding of the lectin *Helix pomatia* agglutinin (HPA) to tumor cells is associated with poor prognosis in clinical studies of colorectal cancer (Schumacher and Adam, 1994). HT29 cells metastasizing in SCID mice are positive for HPA-binding, while HPA non-binding human colorectal cancer cell lines, in general, do not metastasize in SCID mice (Schumacher and Adam, 1997). The aim of the present investigation was to compare the phenotypic and metastatic behavior of the original HT29 (HT29 MDR-1 negative) cell line with the behavior of the MDR-1 overexpressing counterpart when transplanted into SCID mice. Because of the association of HPA binding with metastasis, particular reference will be given to lectin binding sites in these two different variants of HT29.

Materials and methods

Cell culture

The human colon carcinoma cell line HT29 (MDR-1 negative) was obtained from the American Type Tissue Culture Collection through the European Cell Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) and maintained under standard conditions as indicated in the data sheet supplied with the cells. MDR-1 expression was induced by continuous culture in gradually increasing concentrations of colchicine, starting at 2.5 ng/ml, as described previously (Breuer et al., 1993). In order to maintain MDR-1 expression, the MDR-1 positive cells were grown continuously with the selecting drug colchicine (300 ng/ml). Before injection into SCID mice, HT29 MDR-1 positive cells were checked for their functional MDR-1 activity by the rhodamine dye assay (Neyfakh, 1988). The two cell lines were cultured *in vitro* under standard culture cell conditions (37 °C, 100% relative humidity, 5% CO_2) in RPMI medium (Gibco/Life Technologies, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco).

SCID mice

Pathogen-free male and female BALB/c C57BL/KaIgh-I scid/scid (SCID) mice, aged 6–10 weeks, were bred and maintained in filter top cages. Animals housed up to five to a cage were provided with sterile food and water *ad libitum*. A UK Home Office licence had been granted for these experiments and all animal welfare guidelines were adhered to. All experimental manipulations were carried out aseptically inside a laminar flow hood. For injection, the cells were harvested by trypsinization and viable cells (5×10^7) were suspended in 1 ml medium. 200 μl of this suspension was injected subcutaneously between the scapulae of each SCID mouse. Each cell line was injected into groups of between 7 and 10 animals. The tumor development was monitored using electronic calipers and the tumor volume was calculated using the following formula: smallest diameter^2 [mm] × largest diameter [mm] = tumor volume [mm^3]. The experiment was terminated when sizeable tumors had grown (for HT29 MDR-1 negative after 24 days, for HT29 MDR-1 positive at 37 days). The tumors were dissected out, cut into half and fixed in 10% neutral buffered formalin then embedded in paraffin wax.

Histology and histochemistry

5 μm thick sections were cut and stained with hematoxylin and eosin (HE), with a standardized Alcian blue at pH 2.5 (Schumacher and Adam, 1994) and periodic acid-Schiff (PAS) reaction for general mucin histochemistry.

Table 1
Lectins their abbreviation and sugar specificity used in this study.

Origin of the lectin	Abbreviation	Sugar specificity
Helix pomatia	HPA	N-Acetylgalactosamine
Wistaria floribunda	WFA	N-Acetylgalactosamine
Arachis hypogaea	PNA	Galactose
Artocarpus integrifolia	JAC	Galactose
Maclura pomifera	MPA	Galactose
Triticum vulgaris	WGA	N-Acetylglucosamine
Canavalia ensiformis	Con A	Mannose, glucose
Galanthus nivalis	GNA	Mannose
Phaseolus vulgaris	PHA-L	Complex carbohydrates
Phaseolus vulgaris	PHA-E	Complex carbohydrates

Lectin histochemistry

Lectin histochemistry was performed using an indirect technique with biotinylated lectins (for lectins, their abbreviation and sugar specificity see Table 1). All lectins were obtained from Sigma–Aldrich (Poole, Dorset, UK) and an avidin biotin peroxidase complex was used for visualization (ABC Peroxidase, Vector Laboratories, Peterborough, UK). Deparaffinized sections were briefly incubated in Tris-buffered saline (TBS) containing 50 nM Tris, 150 mM NaCl adjusted to pH 7.6 and were than incubated with 0.1% trypsin dissolved in TBS for 15 min at 37 °C. The sections were washed three times in TBS and incubated with the biotinylated lectins (Table 1) for 1 h. The lectins were diluted in lectin buffer (LB, consisting of TBS with 1% $MgCl_2$ and 1% $CaCl_2$ added). After a further three washes in LB, the sections were treated for 30 min with an avidin–horseradish peroxidase complex, prepared according to the manufacturer's instructions. After three further washes, sections were incubated with DAB/hydrogen peroxide prepared according to the manufacturer's instructions for 10 min to visualize the enzyme complex. A light hematoxylin counterstain was used (for methodological details see Schumacher et al., 1995). Control experiments were performed by pre-incubating the lectins with a 0.1 M monosaccharide solutions except for PHA, where bovine thyroglobulin was used as an inhibitor containing complex carbohydrates (for details see Schumacher et al., 2004).

Since indirect immunoperoxidase methods are notoriously difficult to quantify, a direct immunofluorescence method was used to quantify HPA binding. Rehydrated slides were incubated for 30 min at room temperature in a range of concentrations of FITC labelled HPA (5, 10, 15, 20, 50 and 100 μg/ml) in TBS containing 0.2% $CaCl_2$ and washed afterwards three times in the same buffer followed by a wash in distilled water. Slides were mounted in AFI (Citifluor, London, UK) and examined under an Olympus BH5 fluorescence microscope fitted with an Olympus OSP-1 Photometry System. Fluorescence intensity of at least 25 randomly chosen areas from each section was measured using the 40× objective lens. Only enterocyte-like differentiated cells were measured (the strongly HPA positive signet ring carcinoma cells were excluded). A Student's *t*-test was performed using Graph Pad Prism software (Graph Pad Software, La Jolla, CA, USA, Version 2) on an IBM compatible microcomputer. *p*-Values > 0.05 were considered to be statistically significant.

Immunohistochemistry

A similar labelling method was used for immunohistochemistry. Deparaffinized sections were labelled for binding of several monoclonal antibodies using an indirect technique with avidin–peroxidase as an indicator reaction (see Table 2) and hematoxylin as a nuclear counterstain. The dewaxed sections or cytospins with native HT 29 or HT29 MDR positive cells were subjected to antigen retrieval by microwaving the sections in 0.01 M