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Expression of the vascular endothelial cell protein C receptor in epithelial tumour cells

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Abstract

The rat monoclonal antibody LMR-42 has previously been shown to react with an external epitope of a plasma membrane protein with a M_r of approximately 55,000 that was upregulated in multidrug-resistant (MDR) tumour cells. Here, we report the isolation of the cDNA encoding the LMR-42 antigen from the MDR human fibrosarcoma cell line HT1080/DR4 and the lung cancer cell line GLC4/ADR by expression cloning. Sequence analysis showed that the LMR-42 antigen is identical to the endothelial cell protein C receptor (EPCR). Using the LMR-42 Mab for cytochemical analyses of a disease-oriented panel of 45 non-drug selected tumour cell lines of the National Cancer Institute (NCI), we found high *EPCR* expression in 47% of the primary tumour cell lines, including melanomas, renal- and colon carcinomas. In a small panel of human tumours, occasionally very high *EPCR* expression was detected in endothelial vessels, but expression in the tumour cells was a rare event. The functional significance of overexpression of *EPCR* on both primary and drug-selected tumour cells is still unclear. As the protein is related to MHC class I molecules and shares no characteristics with any of the currently known transporter proteins, EPCR is not expected to play a causal role in the resistant phenotype of the MDR tumour cells. Nevertheless, exposure of tumour cells to cytostatic drugs may frequently lead to EPCR overexpression. Since EPCR is known to play a pivotal role in preventing blood coagulation through binding of (activated) protein C, it might endow tumour cells, both of mesenchymal and epithelial derivations, with increased growth potential by local anti-coagulant activity. © 2002 Published by Elsevier Science Ltd.

Keywords: Multidrug resistance; LMR-42 monoclonal antibody; Expression cloning; Endothelial cell protein C receptor; Anticoagulation

1. Introduction

Multidrug resistance (MDR) has been associated with the overexpression of genes involved in metabolism and transport of various anticancer drugs. ''Classical'' MDR is associated with the product of the *MDR1* gene, P-glycoprotein (MDR1 P-gp, ABCB1), reviewed in Ref. [1]. MDR1 P-gp is a plasma membrane protein, that acts as an adenosine triphosphate (ATP)-dependent efflux pump for natural product drugs. MDR1 P-gp overexpression is particularly prominent in tumour cell lines selected *in vitro* by high concentrations of natural product drugs. In several non-P-gp MDR tumour cell lines, high levels of other members of the ATP-binding cassette (ABC) transporter superfamily [2] have been identified. First, the Multidrug Resistance Protein 1 (MRP1, ABCC1; reviewed in Ref. [3] was found to be capable of transporting a range of substrates, including cytotoxic drugs, either or not associated with glutathione [4,5]. In addition, other MRP family members (MRP2–6) [6,7] were identified to contribute to MDR, as well as another novel ABC-transporter, the breast cancer resistance protein (BCRP, ABCG2) [8]. BCRP is a 70 kD transporter protein that probably acts as a (hetero/homo) dimer in transporting (MDR) drugs. This transporter is involved in mitoxantrone and topotecan resistance [9,10].

In an ongoing search for proteins involved in drug resistance, we selected monoclonal antibodies (Mabs) against proteins with elevated levels in non-P-gp MDR cell lines. Immunisation of mice with the lung cancer cell line SW-1573/2R120 led to the development of the LRP-56 monoclonal antibody (Mab) [11]. Expression cloning

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of the corresponding cDNA identified the M_r 110,000 antigen as the human major vault protein (MVP) [12]. The MVP protein constitutes the major component of vault particles, large cytoplasmic ovoid shaped structures with as yet unknown function(s). Still, a connection between the expression of MVP/vaults and MDR has been reported for various different tumour types (see reference [13] and references herein). In another search using rats, we generated a panel of six Mabs reactive with proteins present at high levels in non-P-gp MDR tumour cell lines, but low or absent in the corresponding drug-sensitive, parental tumour cell lines [14]. One of these Mabs, LMR-5, also reacts with the MVP molecule, as shown by staining of *MVP* cDNA transfected MOP8 cells [14]. Another one, the LMR-42 Mab, reacts with a protein of approximately M_r 55,000, upregulated in doxorubicin selected cell lines of different histogenetic origin. Although the epitope recognised by LMR-42 is exposed on viable cells, daunorubicin accumulation of LMR-42-positive tumour cell lines was not affected by saturating concentrations of LMR-42 [14]. In normal human tissues, LMR-42 staining was mainly observed in vascular endothelial cells, next to weak staining of muscle cells and Leydig cells within the testis [14]. In this study, we examined the presence of the LMR-42 antigen in a broad panel of primary and drug-selected human tumour cell lines.

2. Materials and methods

2.1. Cell lines

A total of 45 cell lines of the National Cancer Institute's (NCI) Anticancer Drug Screen Panel was obtained and processed as previously described in Ref. [15]. The panel includes cell lines derived from cancers of the colon, kidney, lung, ovary and brain, as well as melanoma and leukaemia. All other cell lines used have been described elsewhere. The daunorubicin-resistant HT1080/DR4 fibrosarcoma cell line in Ref. [16] and the human small cell lung carcinoma (SCLC) cell line GLC4 and its doxorubicin-resistant, MRP1-positive subline GLC4/ADR in Ref. [17]. The polyoma transformed NIH/3T3 mouse fibroblast cell line MOP8 [18] was used for the transfection experiments.

The resistant sublines were routinely cultured in the presence of doxorubicin until 3–10 days before the experiments. The GLC4 and GLC4/ADR and NCI tumour panel cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium with 10% heat-inactivated fetal calf serum (Gibco Europe, Paisley, Scotland) and 2 mM L-glutamine. The other cell lines were cultured in Dulbecco's modified essential medium (DMEM; Gibco Europe) with the same supplements. All cell lines were passaged once or twice weekly and routinely examined for *Mycoplasma* contamination.

2.2. Tumour samples

Tumour samples were obtained from the tissue bank of the Free University Hospital (Amsterdam, The Netherlands). The tissues were acquired from surgical pathology specimens within 2 h of resection, and autopsy specimens within 12 h of death. All samples were snap-frozen and stored in liquid nitrogen until use.

2.3. Immunocytochemistry

Cytocentrifuge preparations and cryosections (4 μm) were air-dried overnight and fixed for 7 min in acetone. The slides were incubated for 1 h with LMR-42 (1:500) or isotype-matched rat Ig in phosphate-buffered solution (PBS) containing 1% bovine serum albumin (BSA). Mab binding was detected using a biotinylated rabbit anti-rat IgG (1:100) and streptavidin conjugated to horseradish peroxidase (1:500) (Zymed, San Francisco, CA). The colour was developed with 0.02% (w/v) amino-ethyl-carbazole and 0.02% (v/v) H_2O_2 in 0.1 M NaAc pH 5.0. Coded slides were evaluated to avoid any bias in the scoring. For the tumour cell lines, a semi-quantitative "staining index" was calculated as the product of the fraction of positive cells and the average staining intensity estimated on a scale from 0 (negative) to 3 (very strongly positive). At least three tests for each cell line were used for calculation of the average staining index. The parental GLC4 and MDR GLC4/ADR cell lines served as controls for LMR-42 staining.

2.4. COMPARE analysis

The COMPARE program (available at the NCI Developmental Therapeutics Program website (<http://dtp.nci.nih.gov>) was used to examine correlations in the 45 cell lines between the LMR-42 staining results and sensitivity for compounds tested and with basal patterns of gene expression.

2.5. cDNA library

Previously, a cDNA-library was obtained from mRNA isolated from the human non-P-gp MDR fibrosarcoma cell line HT1080/DR4 [12]. Briefly, a size-fractionated (>2 kbp) oligo d(T)-primed cDNA-library was constructed in the shuttle vector pCDM8 using non-self complementary *Bst*X1 adaptors (Invitrogen, Leek, The Netherlands). Transformation of the library into the *Escherichia coli* strain MC1061/P3 by electroporation yielded approximately 100 000 primary colonies. These were divided into 10 sublibraries of 10 000 colonies each.

2.6. Isolation of LMR-42 cDNA clone

Bacterial subpools were grown overnight in Luria-Bertani medium, supplemented with 7.5 μg/ml tetracycline

(Boehringer Mannheim B.V., Almere, The Netherlands), and 12.5 μg/ml ampicillin (Sigma Chemie, Bornem, Belgium). Plasmid DNA, containing cDNA inserts, was isolated from minipreparations of the bacterial sublibraries by alkaline lysis. MOP8 cells were transfected with isolated plasmid DNA using the DEAE-dextran method (Promega Corporation, Leiden, The Netherlands) as described by Aruffo and Seed in Ref. [19]. Transfected MOP8 cells were allowed to grow for 72 h, and after trypsinisation cytospins were prepared. These cytospins were air-dried overnight, fixed in 100% acetone for 7 min and stained with LMR-42 (1:500) to detect transiently expressed protein as described above. A colony containing the cDNA coding for the LMR-42 antigen was isolated by screening progressively smaller pools of bacterial colonies.

2.7. RT-RACE PCR

In order to obtain an overlapping cDNA fragment for verifying the cDNA sequence, a rapid amplification of cDNA ends (RACE) protocol [20] was performed with mRNA isolated from the non-P-gp MDR cell line GLC4/ADR, using RNAzol (Tell-Test, Inc, Friendswood, Texas). One microgramme RNA was heated at 65 °C for 5 min, chilled on ice, and added to 2.0 μl of 10× reverse transcription (RT)-buffer (1×RT buffer contains 50 mM Tris–HCl, pH 8.3, 3 mM $MgCl_2$ and 75 mM KCl), 2 μl 10 mM dithiothreitol, 10 μl 2 mM deoxynucleotite triphosphates (dNTP) at 0.5 mM, 0.5 μl RNasin (Promega Biotec, Madison, WI), 50 pmol of oligo d(T)RACE and 0.2 μl of avian myeloblastosis viral reverse transcriptase [20] and incubated for 45 min at 42 °C. Hot-start polymerase chain reaction (PCR) was performed with RACE-, and internal oligo-primers for 20, 30 and 40 cycles in a thermocycler (Biomed, Theres, Germany). Each cycle consisted of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and chain elongation at 72 °C for 1.5 min. The PCR products (5 μl/lane) were analysed using 1.5% agarose (Biozym, Rockland, ME) gel electrophoresis, and visualised with ethidium bromide staining. The specificity of the PCR product was confirmed by Southern blot hybridisation using a [α-^{32}P]dCTP labelled cDNA probe, obtained as described above from HT1080/DR cDNA expression cloning. The *LMR-42*-specific PCR product was extracted from a duplicate gel by a freeze/squeeze method and phenol/chloroform and isopropanol precipitation, and used for the sequence analysis.

2.8. cDNA sequence analysis

The *LMR-42* cDNA clone and RT-RACE-products were sequenced using the dideoxy Terminator Cycle Sequencing Kit on an automated 373A DNA sequencer (Applied Biosystems Benelux B.V., Maarsen, The Netherlands). In addition to the T7 sequencing primer, three internal sequence-specific synthetic oligodeoxynucleotide primers were used. The labelled DNA fragments were extracted twice with phenol/H_2O/chloroform (v/v/v: 68/18/14), precipitated with ethanol and resuspended in 5 μl denaturant mixture (50 mM ethylenediamine tetra acetic acid (EDTA)/formamide, v/v: 1/5). The samples were boiled for 2 min, chilled on ice, and 4 μl was loaded immediately onto a 6% polyacrylamide sequence gel (acrylamide/bisacrylamide: 19/1; 8.3 M urea). Sequencing was routinely performed in both orientations to confirm the nucleotide sequence. The data were collected and analysed using 373A computer software.

3. Results

3.1. LMR-42 expression in non-selected and drug-resistant tumour cells

As reported earlier in Ref. [14], the LMR-42 Mab reacts with an external epitope of a plasma membrane

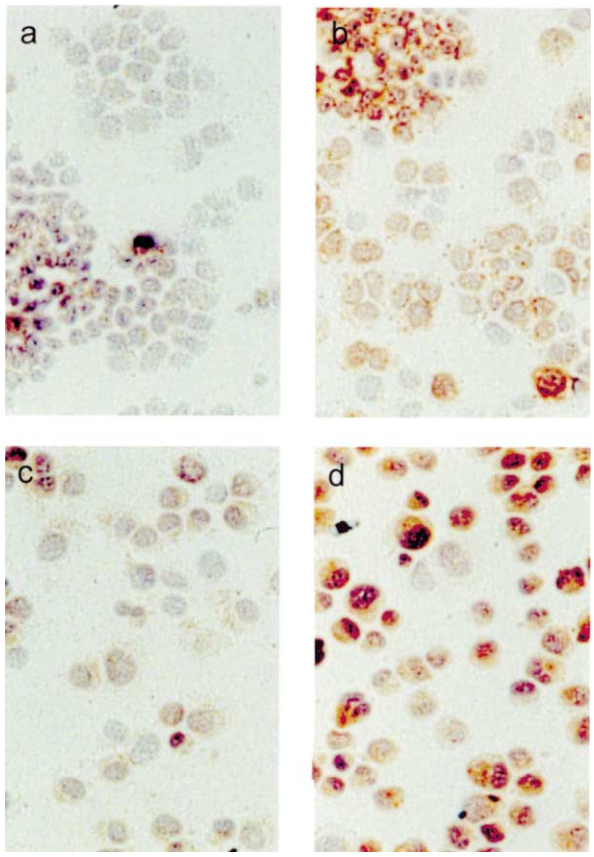

Fig. 1. LMR-42 staining of cytospins of GLC4 (a), GLC4/ADR (b), HT1080 (c) and HT 1080/DR4 cell lines. Increased levels of LMR-42 staining is observed in the multidrug resistant sublines. Although fluorescent activated cell sorting (FACS) experiments on viable cells have shown that the LMR-42 Mab reacts with an external epitope, LMR-42 reactivity in the cytospins may seem to have a more cytoplasmic localisation, due to the nature of the staining technique. Colour was developed with 0.02% (w/v) amino-ethyl-carbazole and 0.02% (v/v) H_2O_2.

antigen. Heterogeneous LMR-42 staining is observed in several different non-P-gp MDR cell lines, while lower or no staining is observed in the parental cell lines. In Fig. 1, the LMR-42 cytospin staining results in the GLC4 and GLC4/ADR cell lines, as well as in the HT1080 and HT1080/DR4 cell lines are shown. Due to the nature of the staining technique, LMR-42 reactivity in the cytospins may seem to have a more cytoplasmic localisation.

To further study the prevalence of the LMR-42 antigen, a panel of drug-unselected human tumour cell lines of the US NCI's anticancer drug screening programme was examined for LMR-42 staining. In 21 out of 45 tumour cell lines of this panel, LMR-42 staining was found (Table 1). Positive staining was particularly strong in distinct tumour types i.e. colon carcinomas (4/9 cell lines), renal cell carcinomas (3/8 cell lines) and melanomas (4/7 cell lines). LMR-42 staining was frequently

Table 1
Expression of the MDR-related proteins P-gp, MRP1, MVP and LMR-42 / EPCR in the NCI disease-oriented panels of human cancer cell lines

Cell line	Tumour type	MDR1 P-gp	MRP1	MVP	LMR-42/ EPCR
HCT-116	Colon	⊙	●●	●●●	●●
COLO-205	Colon	⊙	●●	●●●	●●
KM12	Colon	○	⊙	○	●●
HCC-2998	Colon	○	●●	●●	●
KM20L2	Colon	○	●	●●	⊙
HT29	Colon	○	○	●●	⊙
HCT-15	Colon	●	●●	⊙	○
SW-620	Colon	⊙	●	⊙	○
DLD-1	Colon	●	○	⊙	○
SN12	Renal	○	⊙	●●	●●
TK-10	Renal	○	●●	●●	●
786–0	Renal	⊙	●	●●	●
A498	Renal	○	ND	ND	⊙
UO-31	Renal	⊙	●●	●●●	○
RXF-631	Renal	○	⊙	●●	○
ACHN	Renal	●	○	●●	○
RXF-393	Renal	○	●	⊙	○
M14	Melanoma	○	●	●●	●●
M19-MEL	Melanoma	○	●●	●	●●
SK-MEL-28	Melanoma	○	●	⊙	●
LOX	Melanoma	○	●	○	●
MALME-3M	Melanoma	○	●	●●	○
UACC-257	Melanoma	○	●	⊙	○
SK-MEL-2	Melanoma	○	⊙	⊙	○
NCI-H226	Lung	●	●	●●	●
HOP-62	Lung	○	●●	●●	⊙
NCI-H23	Lung	○	⊙	⊙	⊙
EKVX	Lung	●	⊙	●●	○
HOP-18	Lung	○	ND	●●	○
LXFL-529L	Lung	○	●	●●	○
HOP-92	Lung	○	ND	⊙	○
NCI-H522	Lung	○	⊙	○	○
A549	Lung	○	○	○	○
DMS-114	SCLC	○	●	⊙	○
DMS-273	SCLC	○	⊙	○	○
SF-295	CNS	○	●●	●	●
SNB-78	CNS	○	●	●●	⊙
SNB-75	CNS	○	⊙	●	⊙
SF-539	CNS	○	⊙	●	○
SF-268	CNS	○	●	○	○
XF-498	CNS	○	ND	⊙	○
OVCAR-3	Ovary	○	⊙	○	⊙
OVCAR-8	Ovary	⊙	●●	●●	○
OVCAR-5	Ovary	○	⊙	●●	○
RPMI 8226	Leukaemia	ND	○	●	○

The staining data of MDR1 P-gp, MRP1 and MVP are given for comparison. (MDR1 P-gp adapted from Wu and colleagues [15], MRP1 and MVP adapted from Izquierdo and colleagues [37]. The staining indices were calculated as the product of the fraction of positive cells and staining intensity, and presented as follows: 0 = ○, 0 – 0.5 = ⊙, 0.5 – 1.0 = ●, 1.0 – 2.0 = ●●, 2.0 – 3.0 = ●●●, ND = not determined.

accompanied by MRP1-, MVP- and/or MDR1 P-gp staining. In general, MDR1 P-gp staining was an infrequent event in these cell lines. When the LMR-42 staining values for the tumour cell lines were utilised as a "seed" pattern for the COMPARE program, no correlations greater than 0.4 were observed for the anticancer agents tested. Extensions of these analyses to the whole synthetic database showed that the top three compounds had some structural similarity, but no correlations greater than 0.7 were observed. Likewise, no associations were noted with basal patterns of gene expression. Application of the COMPARE analysis to the molecular targets database demonstrated that the best correlations (ranked number one and two respectively) were with the other MDR markers MVP and MRP1. While these were the best correlations observed, they were not particularly strong, with correlation coefficients in the 0.3–0.4 range.

3.2. LMR-42 expression in human tumour samples

In a panel of 34 tumours comprising paired frozen samples of tumours of different histogenetic origin, LMR-42 expression was examined by immunohistochemical staining (Table 2). Compared with the cell line panel, LMR-42 expression was observed in a more limited number of cases. In several tumour samples, LMR-42 staining was present in endothelial vessels, but was undetectable in the corresponding tumour cells. However, positive LMR-42 staining was observed in a sarcoma sample and in a case of small intestinal cancer. Particularly high LMR-42 staining was observed in the endothelial vessels of a cervical cancer and a kidney cancer (Fig. 2).

Table 2
LMR-42/EPCR levels in frozen sections of human tumours

Tumour type	*n*	LMR-42/EPCR staining	
		Tumour cells	Endothelial cells
Small intestine, adenocarcinoma	1	-	-
Small intestine, adenocarcinoma	1	++	-
Colon, adenocarcinoma	2	+/-	-
Pancreas, adenocarcinoma	2	-	-
Adrenal gland, adenocarcinoma	2	-	-
Stomach, adenocarcinoma	2	-	+
Kidney, Grawitz tumour	1	-	+++
Kidney, Grawitz tumour	1	-	-
Lung, adenocarcinoma	1	+/-	-
Lung, adenocarcinoma	1	-	+
Testis, seminoma	2	-	+
Prostate, adenocarcinoma	2	-	+
Bladder, urothelial cell carcinoma	2	-	-
Mamma, adenocarcinoma	2	-	-
Cervical cancer, squamous cell carc.	1	-	++
Cervical cancer, squamous cell carc.	1	-	-
Ovarian cancer, adenocarcinoma	2	-	-
Melanoma	2	-	-
Round cell sarcoma	1	-	-
Fibrosarcoma	1	+	-
Neuroblastoma	2	-	-
Angiosarcoma	1	-	-
Schwannoma	1	+/-	-

n: Number of samples; carc.: carcinoma; -: no staining; +/-: very weak staining; +: weak staining; ++: positive staining; +++: strong staining.

3.3. Isolation of LMR-42 cDNA

In order to isolate the cDNA sequence of the LMR-42 antigen, a eukaryotic expression cloning system was applied, as successfully used before for the identification of the LRP-56 antigen [12]. In the primary screening of the cytospin preparations of MOP8 cells, transiently transfected with pCDM8 plasmids containing cDNA inserts of the non-P-gp MDR fibrosarcoma cell line HT1080/DR4, a single LMR-42 immunoreactive MOP8 cell was detected among approximately 20×10^6 cells. The sublibrary fraction containing the cDNA of the LMR-42 antigen was selected and the coding cDNA clone was isolated after 9 cycles of screening for the transient presence of LMR-42 antigen in progressively smaller pools of bacterial colonies.

3.4. Sequence analysis of LMR-42 cDNA

Sequence analysis of the *LMR-42* cDNA insert showed that the 760 bp insert contained a single open reading frame, but no in-frame stop codon. To verify the 3′ end of the cDNA sequence, an approximately 700 bp PCR fragment was produced according to the RT-RACE protocol, using mRNA of another, unrelated non-P-gp MDR tumour cell line, the SCLC cell line GLC4/ADR. Specificity of the generated PCR fragment was shown by Southern blot hybridisation with a *LMR-42* cDNA probe (data not shown). From a duplicate gel, the PCR product was isolated and used for sequence analysis. The fragment contained 180 identical overlapping base pairs (bp) at the 3′ end of the coding sequence. An in-frame stop codon was found in the next 20 bp, followed by an additional, apparently non-translated, fragment of 500 bp. The complete nucleotide sequence of the open reading frame was then pieced together and the amino acid sequence was deduced. The coding cDNA sequence contains 714 base pairs, and codes for a 238 amino acid protein with a calculated molecular mass of 27 kDa. The sequence is available under Accession Number X89079 at the EMBL and Genbank databases.

3.5. Homology search

A database search revealed that the LMR-42 antigen is identical to the endothelial cell protein C receptor

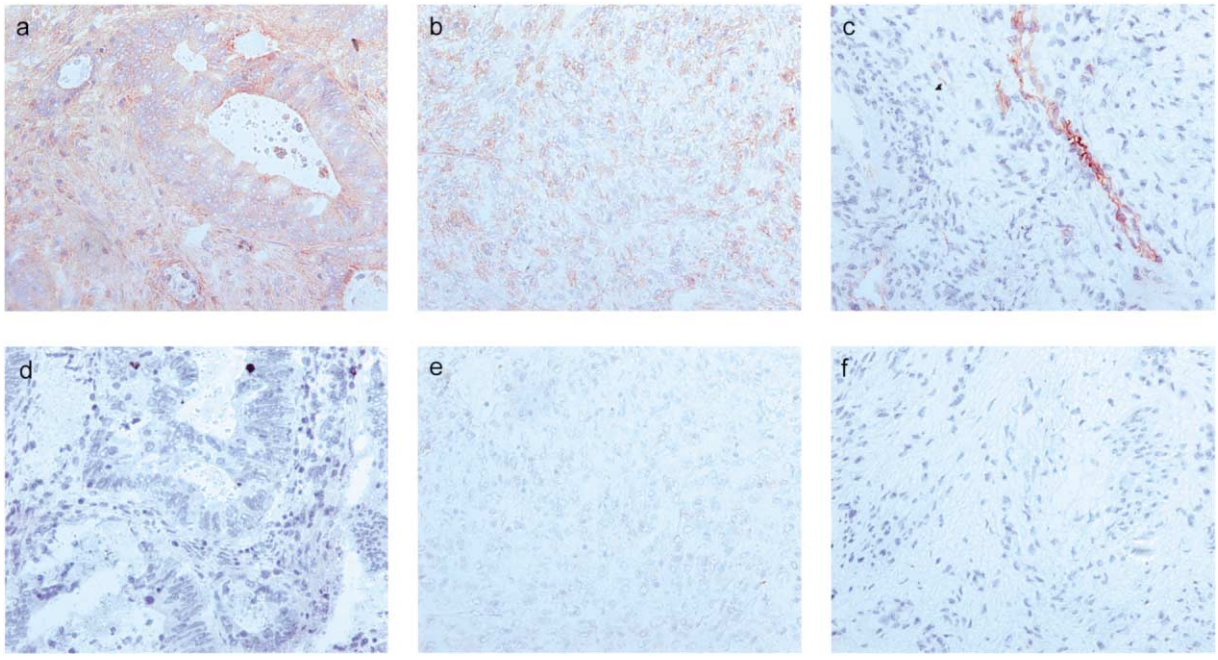

Fig. 2. Staining of frozen sections of tumour samples with LMR-42 / EPCR (upper panel) and control Mab (lower panel). A case of small intestinal cancer (a and d), a fibrosarcoma (b and e) and a case of cervical cancer (c and f) is shown. Positivity of LMR-42 Mab in tumour cells is observed in the first two samples, whereas in the last sample LMR-42 staining is observed only in the endothelial cells. Colour was developed with 0.02% (w/v) amino-ethyl-carbazole and 0.02% (v/v) H_2O_2.

(EPCR) described by Fukudome and Esmon in Ref. [21]. The nucleotide sequence of *LMR-42* was found to differ from the *EPCR* sequence in only one single nucleotide in the transmembrane domain. This difference was observed in the sequence analysis of both the original plasmid insert and in the RACE-PCR product. The base change from G to A led to an amino acid change at position 219 from glycine to serine. Of note, a recent independent *EPCR* database-entry by Simmonds and colleagues [22] showed the same G to A transition that we have observed.

4. Discussion

The antigen of the LMR-42 Mab, with an observed M_r of approximately 55,000, was previously found to be upregulated in different non P-gp MDR tumour cell lines [14]. Here, we observed that this antigen is also frequently present in primary, non-drug selected tumour cell lines (Table 1). In line with the above findings, highest staining levels were observed in cell lines derived from tumours that are generally considered to be poorly responsive to chemotherapy, such as colon carcinomas and renal cell carcinomas, as well as melanomas. In contrast, cell lines considered more sensitive to chemotherapy, such as central nervous system (CNS)-, ovary- and leukaemia cell lines generally showed low levels of LMR-42 staining. However, when analysed with the NCI COMPARE program, no clear correlation between the LMR-42 staining and sensitivity to particular cytotoxic compounds was observed in these cell lines. However, weak correlations were found with LMR-42 staining and staining patterns of the other MDR related molecules, MRP1 and MVP. In a small panel of human tumours, occasionally strong, LMR-42 staining was detected in endothelial vessels, but expression in the tumour cells was a rare event. Interestingly, LMR-42 staining of endothelial vessels in a cervical cancer and a kidney cancer appeared elevated compared with the intensity generally observed in normal tissues.

Collectively, these results suggested the potential relevance of the cognate antigen in clinical drug resistance, and provided the incentive for identification of the gene encoding the LMR-42 antigen.

The corresponding cDNA was cloned using a conventional eukaryotic expression cloning procedure. Since sequencing of the *LMR-42* cDNA clone revealed no in-frame stop codon, additional sequence information was obtained from another cDNA fragment, generated with the RACE technique. The original *LMR-42* cDNA clone obtained by expression cloning may have been truncated by bacterial recombination. Thus, the final sequence obtained resulted from usage of two different, *i.e.* sarcoma and lung carcinoma, tumour cell lines. A database search showed no homology of the LMR-42 antigen with known MDR ABC transporter proteins. Instead, the LMR-42 protein appeared to be

identical to the endothelial cell protein C receptor (EPCR) originally cloned by Fukudome and Esmon [21]. The G to A transition, resulting in a glycine to serine amino acid substitution at position 219, that we observed when sequencing both the original plasmid insert obtained from HT1080/DR4 cells and the GLC4/ADR-derived RT-RACE PCR product, was confirmed in a recent independent *EPCR* database entry by Simmonds and colleagues [22]. Apparently, a serine at that position is a more common event than a glycine. Although the calculated M_r of EPCR is 27,000, EPCR was found to have a M_r of 46,000, due to the presence of carbohydrate moieties located at 4 potential *N*-glycosylation sites [22,23].

EPCR is a type 1 transmembrane glycoprotein containing two domains in the extracellular region that are homologous to the α1 and α2 domains of CD1/MHC class I molecules [22,24]. In human tissues, the protein was found to be present almost exclusively at endothelial cells of large blood vessels [25]. Indeed, LMR-42 staining was also found to be limited to vascular endothelial cells, next to weak staining of muscle cells and testicular Leydig cells [14]. EPCR plays a pivotal role in preventing blood coagulation through binding of (activated) protein C, thereby stabilising the thrombin/thrombomodulin complex and allowing proteolytic cleavage of coagulation factors Va and VIIIa [26–29]. In line with these observations, EPCR was shown to play a key role in the prevention of *E. coli*-mediated sepsis. Inhibition of protein C binding to EPCR, through antibody blocking of the EPCR binding site, converted the response to a sublethal dosage of *E. coli* in baboons, into a lethal one, caused by diffuse intravascular coagulation [30]. Next to membrane-bound EPCR, a soluble form (sEPCR), with a slightly lower M_r (43,000), has been detected in plasma [31]. sEPCR is constitutively released at low levels from the endothelium, but this process can be enhanced by stimuli such as phorbol 12-myristate 13 acetate (PMA) or thrombin [32]. In contrast to EPCR, sEPCR may cause an increased tendency for coagulation, probably through competition for (activated) protein C [27,33]. Thus, the soluble protein may act as a negative feedback system for membrane-bound EPCR. Generally, EPCR may play a modulatory role in vascular responses to inflammation, stress or injury [34]. The present identification of the LMR-42 antigen as EPCR shows that this molecule is not only expressed on endothelial cells, but can also be found in primary as well as resistant tumour cell lines of various epithelial origins and in a limited number of tumour samples. The expression of *EPCR* in tumour cells was confirmed by a recent study of Tsuneyoshi and colleagues [35].

Given the distinct role of vascular endothelial EPCR, it is tempting to speculate that the expression of *EPCR* on tumour cells provides growth opportunities in vivo by maintaining a coagulation-free environment. Thus, the increased risk of thrombosis in advanced cancer patients may hold particularly true for tumours lacking *EPCR* expression. Alternatively, with high expression of EPCR on such tumours, EPCR might be shed as a soluble moiety promoting coagulation-induced tissue damage [27,33]. Whichever reasoning holds true, this would not explain why overexpression of *EPCR* might result from in vitro selection of drug-resistant tumour cells. Although *EPCR* expression can be inducible, e.g. by pro-inflammatory stimuli [32], high *EPCR* expression, as observed in the primary tumour cell lines tested, clearly represents a stable trait. In addition, in the drug-selected cell lines, despite culturing the cells for prolonged periods (up to 8 weeks) without the drug, *EPCR* expression remained high. Since the EPCR protein is a member of the CD1/major histocompatibility complex superfamily [22,24] and bears no characteristics of a transporter protein, it is highly unlikely that the protein is actively involved in transmembranous drug transport. Moreover, the chromosomal location at 20q11.2 [22] argues against co-amplification in an amplicon shared with known MDR transporter genes. All presently known MDR transport proteins have chromosome locations different from chromosome 20. Still, Major Histocompatability Complex (MHC) class I molecules may be upregulated on MDR tumour cells by indirect mechanisms. Izquierdo and colleagues [36] found elevated levels of the ABC transporter associated with antigen processing (TAP) in MDR tumour cell lines to be associated with elevated levels of MHC class I molecules. The TAP molecule determines MHC class I-restricted antigen presentation by mediating peptide translocation over the endoplasmic reticulum membrane. In those studies, evidence was obtained for a concomitant role of TAP molecules in resistance to distinct cytostatic drugs.

In conclusion, the present findings highlight the frequent expression of the EPCR molecule, hitherto only known for its presence on endothelial cells and its critical role in preventing undesirable clotting along the larger blood vessels, on tumour cells of various histogenetic, including epithelial, origins. Moreover, EPCR expression may be particularly prominent after the development of cytostatic drug resistance, pointing to a novel resistance mechanism or, alternatively, intriguing cross-talk between coagulation and resistance pathways.

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