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Subcellular Localization and Distribution of the Breast Cancer Resistance Protein Transporter in Normal Human Tissues[1]

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ABSTRACT

High expression of the *Breast Cancer Resistance Protein (BCRP)* gene has been shown to be involved in resistance to chemotherapeutic drugs. Knowledge of the localization of BCRP protein in normal tissues may help unravel the normal function of this protein. Therefore, we characterized the tissue distribution and cellular localization of BCRP in frozen sections of normal human tissues. For this purpose, we used the recently described monoclonal antibody BXP-34 and another independently developed monoclonal antibody directed against BCRP, BXP-21. Both monoclonal antibodies show specific BCRP plasma membrane staining on cytospins obtained from topotecan- or mitoxantrone-selected cell lines, as well as from *BCRP*-transfected cell lines. Immunoprecipitation experiments using either BXP-21 or BXP-34 yielded a clear M_r 72,000 BCRP band from *BCRP*-overexpressing tumor cells. In the topotecan-selected T8 and mitoxantrone-selected MX3 tumor cell lines, BCRP turned out to be differentially glycosylated. In contrast to BXP-34, BXP-21 is able to detect the M_r 72,000 BCRP protein on immunoblots and is reactive with BCRP in formalin-fixed, paraffin-embedded tissues.

Using BXP-21 and BXP-34, prominent staining of BCRP was observed in placental syncytiotrophoblasts, in the epithelium of the small intestine and colon, in the liver canalicular membrane, and in ducts and lobules of the breast. Furthermore, BCRP was present in veinous and capillary endothelium, but not in arterial endothelium in all of the tissues investigated. In the tissues studied, the mRNA levels of *BCRP* were assessed using reverse transcription-PCR, and these corresponded with the levels of BCRP protein estimated from immunohistochemical staining. The presence of BCRP at the placental syncytiotrophoblasts is consistent with the hypothesis of a protective role of BCRP for the fetus. The apical localization in the epithelium of the small intestine and colon indicates a possible role of BCRP in the regulation of the uptake of p.o. administered BCRP substrates by back-transport of substrate drugs entering from the gut lumen. Therefore, it may be useful to attempt to modulate the uptake of p.o. delivered BCRP substrates, *e.g.*, topotecan or irinotecan, by using a BCRP inhibitor. Clinical trials testing this hypothesis have been initiated in our institute.

INTRODUCTION

Drug transporters other than P-gp[4] or the MRP family may be important for resistance to anticancer drugs. Recently, elevated expression of the *BCRP* (*ABCG2*) gene, a member of the ATP-binding cassette transporter family, has been described in drug-resistant ovary, breast, colon, gastric cancer, and fibrosarcoma cell lines (1–5). BCRP has only one ATP-binding cassette and six putative transmembrane domains, suggesting that BCRP is a half-transporter, which may function as a homodimer or heterodimer. However, the identity of a putative partner protein is unknown as yet.

For many drug transporters, a normal physiological role is known. For instance, P-gp is highly expressed in the blood-brain barrier, the intestine, and the placenta and has a protective function for the brain and the fetus by extruding toxic agents (6). MRP1 also appears to function as an outward pump for xenobiotics (7) and contributes to the blood-cerebrospinal fluid barrier (8, 9). For BCRP, the normal physiological function has not been established as yet. In normal human tissues, high expression of *BCRP* mRNA has been noted in the placenta (1, 4). Furthermore, although results between previous studies (1, 4) differ somewhat, low expression of *BCRP* in liver, small intestine, colon, ovary, kidney, and heart was reported. This expression profile allows speculation on a role of BCRP, like P-gp, in protection of the fetus and in the regulation of transport of chemicals through the epithelium of the gastrointestinal tract. The mRNA expression of *BCRP* has been assessed in tissue extracts, but it has not yet been determined in which cell types *BCRP* is expressed and what the subcellular localization of BCRP is.

We have developed recently the Mab BXP-34 directed against human BCRP (10). In this study, we describe a newly developed Mab directed against BCRP, BXP-21. We have used the BXP-21 and BXP-34 Mabs to characterize the tissue distribution and subcellular localization of BCRP. Moreover, for a number of tissues we isolated mRNA from the same blocks for semiquantitative RT-PCR evaluation of the *BCRP* expression levels. Knowledge of the normal tissue distribution may add to the understanding of the normal function of BCRP and may be valuable for future clinical purposes.

MATERIALS AND METHODS

Cell Lines and Tissues. The following cell lines were used in this study: the human IGROV1 ovarian carcinoma cell line, the topotecan-selected subline T8, and the mitoxantrone-selected subline MX3. The T8 and MX3 cells are resistant to topotecan and mitoxantrone and concomitantly show overexpression of the *BCRP* gene (3). Furthermore, a partially revertant T8 cell line (3), the doxorubicin-resistant human GLC4/ADR small cell lung cancer cell line (11), the MCF-7 breast carcinoma cell line, the doxorubicin-selected subline MCF-7 Dox40, the mitoxantrone-selected subline MCF-7 MR (12), as well as the myeloma cell line 8226, the doxorubicin-selected subline 8226 Dox40, and the mitoxantrone-selected subline 8226 MR20 (13) were used. The ovarian carcinoma cell line 2008 and the *MRP1*- and *MRP2*-transfected sublines were described by Kool *et al.* (14) and Scheffer *et al.* (15), and the monkey kidney CV-1 cells, transformed by an origin-defective mutant of SV40 that codes for wild-type T antigen COS7 cells, by Gluzman (16). For control purposes, a dog-kidney-derived *MDR1 P-gp*-transfected cell line, MDCKII/MDR1 (17), was used. All of the cell lines were cultured in RPMI 1640 or DMEM supplemented with 10% FCS, 25 mM HEPES, 110 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were kept in continuous logarithmic growth at 37°C in a humidified atmosphere of 5% CO_2 in air. Normal human tissues were obtained from the tissue cryobank of The Netherlands Cancer Institute or the Free University in Amsterdam. Normal human tissue was obtained from surgical or autopsy specimens. All of the tissues were snap frozen in liquid nitrogen and subsequently stored at −70°C. Blood cells were obtained from

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[4] The abbreviations used are: P-gp, P-glycoprotein; AEC, 3-amino-9-ethylcarbazole; ECL, enhanced chemiluminescence; BCRP, Breast Cancer Resistance Protein; RT-PCR, reverse transcription-PCR; HRP, horseradish peroxidase; Mab, monoclonal antibody; MDR, multidrug-resistance; MRP, MDR Protein; PBGD, porphobilinogen deaminase; PBS/BSA, 1% BSA in PBS; nt, nucleotide.

healthy volunteers. Fresh bone-marrow progenitor cells were from a neuroblastoma patient and were obtained via the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, the Netherlands).

Immunization and Mab Production. The development of the Mab BXP-34, directed against BCRP, has been described elsewhere (10). An independently developed Mab, BXP-21, was raised in Balb/C mice analogous to described methods (10, 18) by injection with a fusion protein consisting of the *Escherichia coli* maltose-binding protein and a 126 amino acids part of the BCRP peptide [amino acids 271-396 of BCRP (GenBank accession no. AF098951)]. This fusion protein was made using the plasmid vector pMal-c (19). The fusion protein was produced in *Escherichia coli* DH5α and purified by amylose resin affinity chromatography (19). The mice were housed and treated according to current regulations and standards of the Institutional Animal Ethics Committee.

The mice were killed, and subsequently draining popliteal lymph nodes were removed and used for fusion with mouse myeloma Sp2/0 cells, as described previously (14). Hybridoma supernatants containing Mabs were first screened on ELISA plates, coated with the above-mentioned fusion protein, or coated with an irrelevant fusion protein as negative control. BCRP fusion protein-positive cultures were screened on octo-spins containing eight cytospins of a mixture of MCF-7 MR and MCF-7 parental tumor cells. Slides were stained as described below. Hybrid cells that secreted antibodies of interest were selected and subcloned three times by limiting dilution. The isotype was determined using Iso*Strips* (Boehringer Mannheim).

Western Blotting. Cells were scraped and subsequently lysed in hypotonic lysis buffer, consisting of 100 μM KCl, 2 μM $MgCl_2$, 100 μM Tris-HCl (pH 7.4), 1% SDS, supplemented with protease inhibitors ("Complete"; Roche Diagnostics, Germany). Lysates were sonicated and stored at −80°C. Protein levels were determined using the Lowry method. Proteins were separated on a 7.5% polyacrylamide gel and subsequently transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Proteins were hybridized using BXP-21 (1:50) and HRP-conjugated goat antimouse IgG (1:1000; Dako, Glostrup, Denmark). For control purposes, P-gp, MRP1, and MRP2 were hybridized using C219 (20), MRPr1 (18), and M_2III-6 (21) as primary antibodies, respectively. Subsequently, proteins were visualized using ECL (Amersham Life Sciences, 's-Hertogenbosch, the Netherlands).

Immunoprecipitation. Cells were preincubated for 2 h with RPMI without methionine and without FCS before labeling. Cells were labeled overnight with 4 μCi/ml [^{35}S]methionine (Amersham Life Sciences) in RPMI without methionine with 10% FCS. Cells were homogenized in lysis buffer (PBS, 1% NP40, 1 mM EDTA, and "Complete" protease inhibitors) for 30 min on ice. Subsequently, nuclei and large debris were removed by centrifugation for 1 min at 14,000 rpm. Supernatants containing approximately 5×10^5 cpm were diluted with lysis buffer and incubated for 1.5 h at 4°C with 2 μg BXP-34 or BXP-21. Immune complexes were precipitated by the addition of 100 μl of 10% protein A-Sepharose in PBS (CL-4B; Pharmacia, Uppsala, Sweden) and incubation at 4°C for 1 h. Precipitates were washed twice with lysis buffer containing 2% BSA and four times with PBS. Next, samples were taken up in 50 μl of sample buffer [final concentrations, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 3% SDS, 5% 2-mercaptoethanol, and 0.05% bromphenol blue]. Protein A-Sepharose was removed by centrifugation, and samples were loaded on a 7.5% polyacrylamide gel. Gels were stained with Coomassie Blue (BluePrint Fast-PAGE Stain; Life Technologies, Inc.) and incubated with NAMP100 amplifier (Amersham Life Sciences). Subsequently, gels were analyzed using a phosphorimaging system (Fujix Bas 2000; Fuji Photo Film Co. Ltd., Tokyo, Japan).

Deglycosylation. Half of a batch of cell lysates of [^{35}S]methionine-labeled T8 and MX3 tumor cells, as prepared for immunoprecipitation, were incubated with 2 units of peptide-*N*-glycosidase F (Roche Diagnostics Nederland B.V., Almere, the Netherlands) at 37°C for 1 h, and the other half was mock-treated under identical conditions. Subsequently, samples were treated as described above for the immunoprecipitation, and gels were analyzed using the phosphorimager.

Immunohistochemistry. Cryostat sections (4 μm) were cut, dried overnight at room temperature, and fixed in acetone for 8 min at room temperature. Cytospin preparations of tumor cells were also fixed in acetone. Formaldehyde-fixed paraffin-embedded tissues were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked using 0.3% (v/v) H_2O_2 in methanol for 20 min. Before staining, paraffin sections were pretreated with 10 mM citric acid (pH 6.0) for 20 min. The slides were first incubated with 5% normal goat serum/PBS for 30 min. Subsequently, frozen sections and cytospins were incubated for 60 min at room temperature with a 1:150 or 1:100 dilution of BXP-21 or BXP-34 hybridoma supernatant, respectively, whereas paraffin sections were incubated with a 1:150 or 1:100 dilution of BXP-21 or BXP-34, respectively, at 4°C overnight. BXP-21 and BXP-34 were diluted in PBS/BSA.

Two staining methods were applied. In the first method, biotinylated goat antimouse IgG (Dako; 1:200) and HRP-conjugated streptavidin (both in 90% PBS/BSA + 10% normal human serum) were used as secondary reagents. Color development was performed using 0.4 mg/ml AEC. The second method used the tyramide/FITC amplification method, as described by De Vree *et al.* (22). In this case, after incubation with the BXP-34 or BXP-21 Mabs, slides were incubated for 60 min with HRP-conjugated goat antimouse IgG (Dako; 1:100 in 90% PBS/BSA + 10% normal human serum), subsequently incubated for 10 min with 1:100 tyramide/FITC in amplification buffer (NEN Life Science Products, Boston, Massachusetts), and finally incubated for 60 min with HRP-conjugated rabbit anti-FITC (Dako; 1:100 in PBS/BSA). Color development was achieved using AEC. After counterstaining with hematoxylin, slides were mounted.

For each type of tissue, negative controls were included, *i.e.*, by omission of the primary Mab, by using the irrelevant IgG1 Mab MOPC 21 (ICN Pharmaceuticals, Aurora, Ohio) or the IgG2a Mab PI 17 (American Type Culture Collection, Manassas, Virginia).

***BCRP* Expression in Cell Lines and Normal Human Tissue.** Poly(A)$^+$ RNA was isolated from cell lines or from 30 × 30-μm cryosections/tissue sample using RNAzol, according to the manufacturer's description. mRNA aliquots (3 μg) were used for semiquantitative RT-PCR. The *PBGD* (NM000190) gene was used as an internal standard. This housekeeping gene was selected, because it is expressed independently of the cell cycle (23, 24). The following primers were applied: 5′-caaccattgcatcttggctg-3′ (forward, nt 1914-1933) and 5′-caaggccacgtgattcttcc-3′ (reverse, nt 2118-2137) for *BCRP* and 5′-tctggtaacggcaatgcggc-3′ (forward, nt 31-50) and 5′-ccagggcatgttcaagctcc-3′ (reverse, nt 264-283) for *PBGD*. For each RNA sample, 12 reactions were performed using 14, 16, and 18 to 36 cycles. Water was amplified for a total of 36 cycles as a negative control. DNA was labeled using [α-^{32}P]dCTP, and products were separated electrophoretically on a 6% polyacrylamide gel. DNA bands were quantified using a phosphorimaging system. Finally, the relative expression of *BCRP* as compared with that of *PBGD* (*BCRP/PBGD*) was calculated using the method of de Lange *et al.* (25). Briefly, at least four intensities of bands were measured in the log-linear part of the amplification cycle *versus* signal intensity curves, and subsequently the distance between the curves of the *BCRP* gene and that of the *PBGD* gene was used to calculate the relative expression levels. The assay was performed in duplicate. Poly(A)$^+$ RNA from the human ovarian IGROV1 and the human small cell lung cancer cell line GLC4-ADR (both having very low levels of *BCRP*), the *BCRP*-overexpressing cell line T8 (high level of *BCRP*; Ref. 3), and the partial revertant T8-40 (intermediate levels of *BCRP*; Ref. 3) was used as control.

RESULTS

The development of the BXP-34 Mab directed against human BCRP was described elsewhere (10). The antibody is capable of staining cell membranes of *BCRP*-overexpressing cell lines, *e.g.*, MCF-7 MR (12), 8226 MR20 (26), T8, and MX3 cells (3). BXP-21 was developed using fusion proteins containing aa 271-396 of BCRP. BXP-21 was shown to be of IgG2a subclass. Incubation of cytospins of *BCRP*-overexpressing cells with BXP-21 yielded a clear plasma membrane staining, as shown for the topotecan-selected T8 cell line in Fig. 1. Specificity of the antibody was further demonstrated using *BCRP*-transfected monkey kidney COS7 cells, as described previously for BXP-34 (10; data not shown). BXP-34 was shown before to not be suitable for Western blotting of BCRP (10). However, by using BXP-21, a band of approximately M_r 72,000, corresponding to the predicted molecular weight of BCRP, was detected in Western blots of *BCRP*-overexpressing tumor cell lines (Fig. 2). Relative levels of

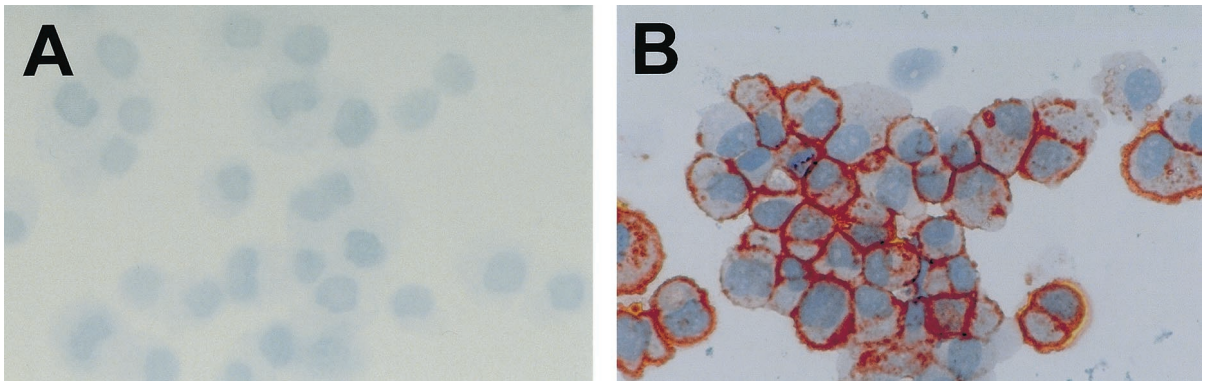

Fig. 1. Staining of cytospins (×100) of the parental IGROV1 ovarian tumor cell line (*A*) and the drug-selected *BCRP*-overexpressing subline T8 (*B*) with BXP-21. Color development was with AEC.

BCRP correlated with known mRNA expression levels of *BCRP* in the cell lines studied (2, 3). BXP-21 did not cross-react with the known MDR transporters P-gp, MRP1, and MRP2 in cytospin preparations (data not shown) and in Western blots (Fig. 3). Immunoprecipitation experiments using BXP-21 or BXP-34 Mabs resulted in bands of approximately M_r 72,000, whereas no bands were detected at this position using an isotype-matched irrelevant antibody (Fig. 4*A*). Interestingly, the molecular weight of the bands in the T8 and MX3 differed slightly in both Western blots and immunoprecipitation experiments. Treatment with peptide-*N*-glycosidase F yielded bands with an equal, slightly lower, molecular weight of approximately 62,000 in both cell lines, showing that this difference was caused by different levels of glycosylation in these cell lines (Fig. 4*B*).

In this study, we assessed the subcellular localization and distribution of BCRP in normal human tissues using BXP-21 and BXP-34 Mabs. BXP-21 Mab is suitable for staining paraffin-embedded, formalin-fixed tissue for BCRP, but BXP-34 did not yield specific staining (data not shown). In this study, therefore, we used frozen sections of normal human tissues to allow comparative analysis using the two different Mabs. The BXP-34 Mab was used for all of the stainings, and a number of tissues was also stained using BXP-21. As controls for staining, *BCRP*-negative IGROV1 and *BCRP*-overexpressing T8 cell cytospin preparations were used. Pronounced staining with antibodies to BCRP was observed in the topotecan-selected cell line T8, moderate staining was observed in the partial revertant T8, and no staining was observed in the IGROV1 and GLC4/ADR cells (Table 1; Fig. 1). In several normal human tissues, strong immunoreactivity was observed (Table 2). Placental tissue showed apical staining of the syncytiotrophoblasts (Fig. 5*A*), whereas liver was strongly stained at the bile canalicular membrane (Fig. 5*B*). Prominent staining was observed in the gastrointestinal tract, with strong apical staining of the epithelium of the small intestine and colon (Fig. 5, *C* and *D*, respectively), and at the apical side in a proportion (but not all) of the ducts and lobules of the breast (Table 2). Furthermore, in almost all of the tissues tested, staining of the venous and capillary endothelial cells was observed (*e.g.*, Fig. 5, *E* and *F*). In contrast, only sporadic staining for BCRP was observed in arterial endothelium (Fig. 5, *G* and *H*). No significant staining was observed in blood cells, *i.e.*, erythrocytes, leukocytes, and platelets.

To compare mRNA expression levels with protein levels of BCRP, semiquantitative RT-PCR was used to determine mRNA levels of *BCRP* in various types of tissue. The *BCRP/PBGD* ratios determined

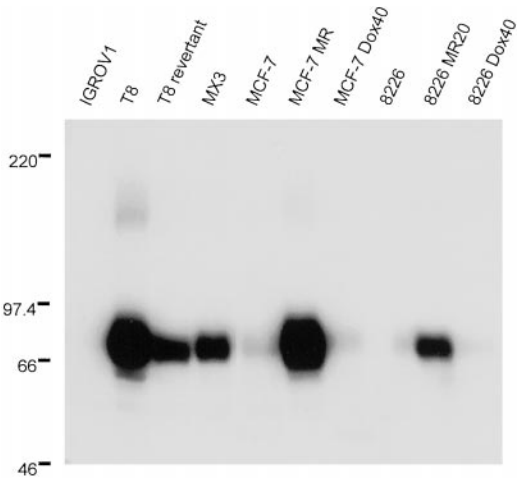

Fig. 2. Western blot analysis of BCRP in total cell lysates from parental (IGROV1, MCF-7, and 8226), *BCRP*-overexpressing resistant (T8, T8 revertant, MX3, MCF-7 MR, and 8226 MR20), and *BCRP*-negative resistant tumor cell lines (MCF-7 Dox40 and 8226 Dox40). BCRP was demonstrated using BXP-21 (1:150) as primary and goat antimouse IgG (1:1000) as secondary reagent. Bands were visualized using ECL. The M_r 200,000 bands are most likely the result of aggregation attributable to the denaturation step. Upon longer exposure times, this band was also visible in all of the other BCRP-expressing cell lines.

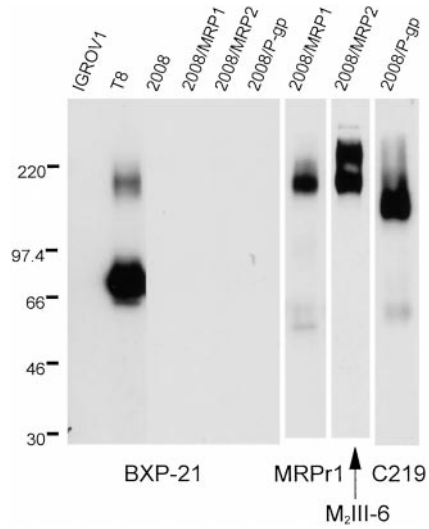

Fig. 3. Western blot analysis of BCRP, MRP1, MRP2, and MDR P-gp in total cell lysates from parental IGROV1 and *BCRP*-overexpressing tumor cell lines. The *BCRP*-overexpressing T8 tumor cells were used, as well as *MRP1*, *MRP2*, and *MDR1 P-gp*-transfected cell lines, 2008/MRP1, 2008/MRP2, and MDCKII/MDR1, respectively. Blots were hybridized using BXP-21, MRPr1, M_2III-6, and C219 as primary antibodies. BXP-21 did not cross-react with MRP1, MRP2, or MDR1 P-gp. Bands were visualized using ECL. Notably, the BXP-21-hybridized lanes were cut from the same blot with identical exposure of the film for all of the lanes. BCRP levels in the 2008 parental tumor cell line are lower than those observed in the IGROV parental tumor cell line.

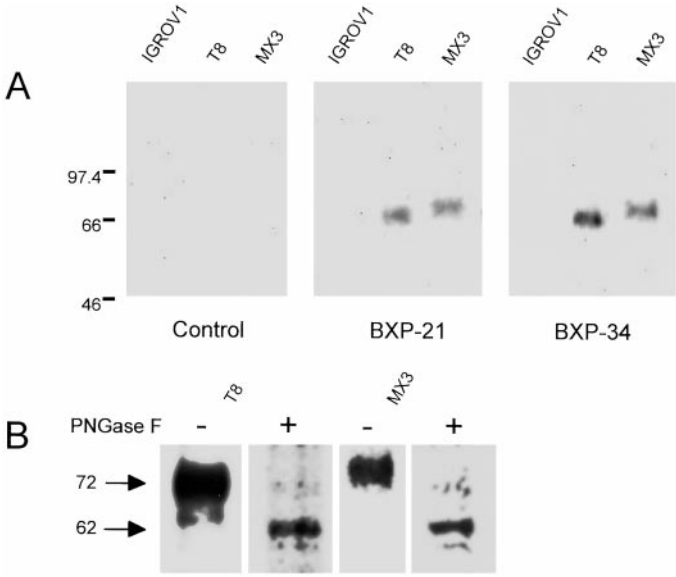

Fig. 4. *A*, immunoprecipitation of BCRP from the IGROV1, T8, and MX3 cells. Cells were labeled using [^{35}S]methionine, as described in "Materials and Methods." BCRP was precipitated using the BXP-21 or BXP-34 Mab. As control for precipitation, mouse antihuman CD68 antibody was used. Gels were analyzed using a phosphorimaging system. *B*, immunoprecipitation of BCRP from the T8 and MX3 cells after treatment with 2 units of peptide-*N*-glycosidase F (*PNGase F*) at 37°C for 1 h (+) or mock-treated (−). Before this treatment, cells were labeled using [^{35}S]methionine, as described in "Materials and Methods." BCRP was precipitated using the BXP-21 Mab. Gels were analyzed using a phosphorimaging system.

Table 1 *Immunohistochemical detection of BCRP using the BXP-21 and the BXP-34 Mabs and BCRP/PBGD mRNA expression ratios in human tumor cell lines*

Tumor cell lines	BXP-21	BXP-34	*BCRP/PBGD*
IGROV1	○[a]	○	0.04
T8	●●	●●	5
T8-revertant	●/○	●/○	1
GLC4/ADR	○	○	0.01

[a] ○, no staining; ●●, strongly positive staining on plasma membrane; ●/○, heterogeneous staining, *i.e.* approximately 15% of the cells positive on plasma membrane, all of the other cells negative.

for the reference cell lines correlated with the known expression levels of *BCRP*, *i.e.*, low levels in the IGROV1 and GLC4-ADR, high levels in the T8, and intermediate levels in the T8-revertant (Table 1). Using this assay, we confirmed previously reported (1, 4) high expression of *BCRP* in the placenta (*BCRP/PBGD* ratio of 4.2, as compared with 5.0 in the T8 cell line). In general, results from the RT-PCR assay were consistent with the immunohistochemical staining. Because of blood vessel endothelial expression of *BCRP*, mRNA levels of *BCRP* were partly influenced by the density of blood vessels in the respective tissues; *e.g.*, low levels of immunohistochemical staining in the heart and breast correlated with low *BCRP/PBGD* ratios (0.26 and 0.85, respectively). High levels of *BCRP* mRNA in the cervix and ovary (ratios, 3.25 and 5.40, respectively) corresponded with extensive immunohistochemical staining of many blood vessels in the tissue sections used in this study (for ovary, see Fig. 5*F*). Furthermore, the relatively large epithelial (villous) surface in the small intestine sections corresponded with a higher *BCRP/PBGD* ratio than in the colon sections (ratios, 3.85 and 1.60, respectively).

DISCUSSION

We have analyzed the normal human tissue distribution of BCRP using a recently developed Mab BXP34 (10) and a newly developed Mab BXP-21. Both Mabs show pronounced staining at the plasma membrane of *BCRP*-overexpressing tumor cell lines. In contrast to BXP-34, BXP-21 is also reactive on immunoblots, yielding a protein band of approximately M_r 72,000, the expected size of BCRP. BXP-21 did not cross-react with other MDR transporters like P-gp, MRP1, and MRP2. In the Western blotting and immunoprecipitation experiments, BCRP was found to be differentially glycosylated in T8 and MX3 tumor cells. Whether this difference in glycosylation reflects differences in BCRP functioning remains to be investigated.

From previously reported mRNA data (1, 4), *BCRP* is known to be highly expressed in the placenta and at lower levels in the liver, small intestine, colon, and ovary. In one study (4), low expression of *BCRP*

Table 2 *Immunohistochemical detection of BCRP in frozen sections of normal human tissue using the BXP-21 and BXP-34 Mabs and BCRP/PBGD mRNA expression ratios in these tissues*

Human tissues		BXP-21	BXP-34	*BCRP/PBGD*
Digestive system				
Esophagus	Epithelium	n.d.[a]	○	n.d.
Stomach	Epithelium	○	○	0.45
Small intestine	Epithelium	●	●	3.85
Colon	Epithelium	●●	●●	
Muscular layers		○	○	1.60
Ganglion cells		○	○	
Liver	Hepatocytes	○	○	
	Bile canaliculi	●	●	2.25
	Bile ductules	○	○	
Pancreas	Acini	n.d.	○	
	Ducts	n.d.	○	0.85
	Islet of Langerhans	n.d.	○	
Excretory system				
Kidney	Glomeruli	○	○	1.65
	Tubules	○	○	
Urinary bladder	Epithelium	n.d.	○	1.00
	Muscularis	n.d.	○	
Male reproductive system				
Prostate gland	Epithelium	n.d.	○	0.70
	Fibromuscular stroma	n.d.	○	
Testis				
Interstitial tissue	Leydig cells	n.d.	○	2.20
Seminiferous tubules	Sertoli cells	n.d.	○	
	Spermatogenic cells	n.d.	○	
Female reproductive system				
Mammary gland (breast)	Lobules	●/○	●/○	0.85
	Lactiferous ducts	●/○	●/○	
Cervix	Epithelium	○	○	3.25
	Cervical glands	○	○	
Ovary	Follicular cells	○	○	
	Germinal cells	○	○	5.40
	Luteinized stromal cells	○	○	
Placenta	Cytotrophoblast	○	○	4.20
	Syncytiotrophoblast	●	●●	
Lymphatic cells				
Spleen	White pulp	n.d.	○	n.d.
	Red pulp	n.d.	○	
Nervous system				
Brain	Parenchyma		○	n.d.
Choroid plexus	Epithelium	○	○	
Periferal nerve		○	○	
Endocrine system				
Adrenal	Cortex	n.d.	○	
	Medulla	n.d.	○	
Cardiovascular system				
Heart	Myocardium	n.d.	○	0.26
Blood vessels				
Arteries	Endothelium	○	○	n.d.
	Tunica media	○	○	
Veins	Endothelium	●	●	n.d.
	Tunica media	○	○	
Capillaries		●	●	n.d.
Blood cells				
Erythrocytes		○	○	n.d.
Leukocytes		○	○	n.d.
Platelets		○	○	n.d.
Progenitor cells		n.d.	●/○	n.d.

[a] n.d., not determined; ○, no staining; ●●, strongly positive staining; ●, positive staining; ●/○, heterogeneous staining, *i.e.* some cells positive, most cells negative. Notably, staining in the endothelium of veins and capillaries in respective tissues is not included in the tissue-staining results. Poly A^+ RNA was collected from the whole tissue section, as indicated with a bar, when appropriate.

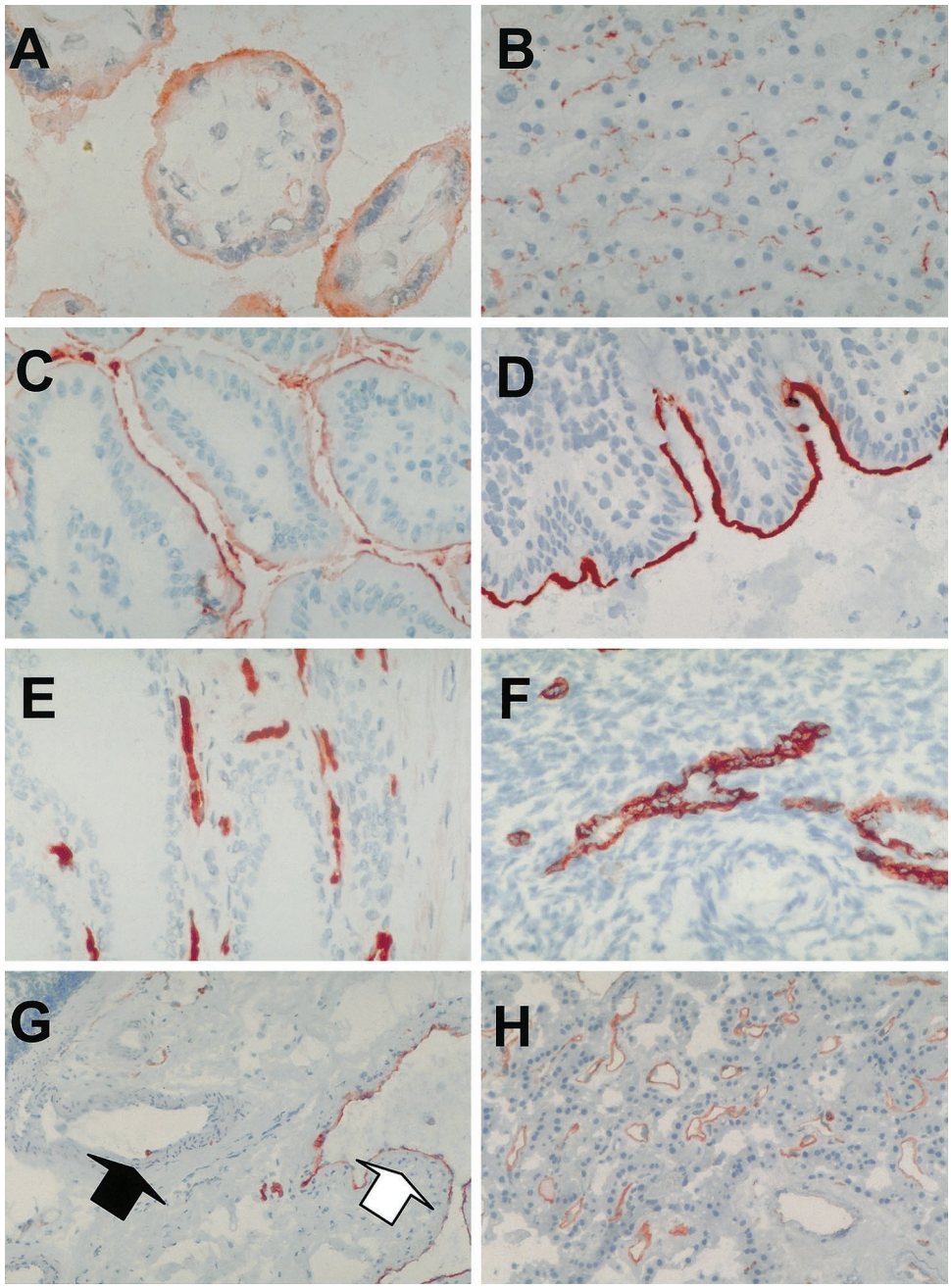

Fig. 5. BCRP staining of cryosections of human tissue using the BXP-21 (*D* and *H*) or BXP-34 (*A–C*, *E–G*) Mab. *A*, placenta (×100); *B*, liver (×100); *C*, small intestine (×100); *D*, colon (×100); *E*, prostate (×100); *F*, ovary (×100); *G*, vein (*white arrow*) and artery (*black arrow*) in the submucosa of the stomach (×33); *H*, choroid plexus (×100). Color development was with AEC.

was observed in the kidney and heart as well. Using semiquantitative RT-PCR, we confirmed the expression of *BCRP* in these tissues, although the relative levels differed. These differences may be caused by the sections of the respective tissues that were used in these studies. Notably, in contrast to the study of Allikmets (4), the expression level of *BCRP* in the heart, as determined by our semiquantitative RT-PCR assay, was one of the lowest of all of the tissues tested, in line with our staining results in the heart (Table 2). Furthermore, our RT-PCR data showed that low to moderate levels of *BCRP* mRNA are present in every tissue tested. This background level of *BCRP* was explained by immunohistochemical staining, which showed BCRP to be present in endothelium of veins and capillaries. The importance of endothelial expression of *BCRP* for the observed tissue expression levels of *BCRP* is illustrated by our results in ovary and cervix tissue. These tissues contain many blood vessels and indeed showed very high expression of *BCRP* for the ovary, even higher than that found in placental tissue (Table 2).

Nonendothelial staining for BCRP was observed in only a limited number of tissues, *i.e.*, the placenta, small intestine, colon, liver, and breast. The presence of BCRP in placental syncytiotrophoblastic cells indicates that BCRP may have a protective function for the fetus. Interestingly, both in wild-type and in mdr1a/1b(−/−) mice, inhibition of mouse Bcrp1 by GF120918, a recently described inhibitor of human BCRP and mouse Bcrp1 (27, 28), resulted in at least 2-fold increased uptake of p.o. administered BCRP substrate topotecan in the fetus (29). Notably, relative mRNA levels of mouse *Bcrp1* in the placenta are lower than that observed in humans; therefore, the protective effect in humans may well be stronger than that observed in mice.

Furthermore, prominent apical staining of BCRP was observed in the epithelium of both the small intestine and the colon, as well as in the canalicular membranes of the liver. The staining in the liver canalicular membrane indicates that BCRP may be involved in excretion processes in the liver, similar to many other ABC transporters,

e.g., P-gp and MRP2 (30). The presence of BCRP in the small intestine and colon suggests that BCRP is involved in the regulation of uptake of substrates from the gastrointestinal tract by back-transport of substrates entering from the gut lumen. This hypothesis is strengthened by the significantly increased plasma levels of p.o. administered topotecan in wild-type or mdr1a/1b (−/−) mice in the presence of the BCRP inhibitor GF120918 (29). Staining in the breast was observed at the apical side of some ductal epithelial cells. Staining for MRP1 has also been observed in this cell type (31). The function of these transporters at this location remains subject to additional investigations.

Finally, BCRP was present in the endothelial layer of veins and capillaries in all of the tissues. At this moment, it is not known if BCRP contributes to transport across the endothelium. The endothelium is known to be quite permeable for several substances, which are known to pass through the endothelial layer between loosely connected endothelial cells. The endothelium in the brain is different in that the endothelial cells form tight junctions, creating the blood-brain barrier. Therefore, expression of transporter proteins at the blood-brain barrier may contribute to protection of the brain. For P-gp, such a specific role in the blood-brain barrier is well established (6). Studies investigating the influence of BCRP in the blood-brain barrier in mice are currently ongoing in our laboratory.

The knowledge of the normal tissue distribution of BCRP, as described in this report, allows attempts to increase exposure of certain tissues. One clinical implication of our findings is that p.o. administration of BCRP substrates, *e.g.*, topotecan and irinotecan (3), may be more efficient when combined with an inhibitor of BCRP. This concept has been proven in mice and patients for paclitaxel, which has low oral bioavailability because of its high affinity for P-gp (32), when administered p.o. combined with the effective P-gp blocker cyclosporin A (33, 34). It is well known that oral bioavailability of topotecan is relatively low and variable (30 ± 7.7%) in patients (35). Considering previously obtained results in mice (29) and the presence of BCRP in the small intestine and colon of humans as reported in this study, this bioavailability can possibly be improved by combining oral topotecan with a BCRP inhibitor, such as GF120918 (28). We have recently started clinical trials aiming at testing the feasibility of this approach. If feasible, this principle may work for other BCRP substrate drugs as well.

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