Multidrug Resistance Protein (MRP) Expression in Retinoblastoma Correlates with the Rare Failure of Chemotherapy despite Cyclosporine for Reversal of P-Glycoprotein

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Abstract

Failure of chemotherapy associated with expression of the multidrug resistance protein p170 frequently occurs in retinoblastoma (RB). Despite using cyclosporine, which inhibits p170 and improves our chemotherapy results, rare failures occur. In nonmetastatic primarily enucleated RBs, we show expression of p170 in 3 of 18 samples and expression of multidrug resistance protein (MRP), the second protein associated with resistance to chemotherapy, in 1 of 18 samples. All three RBs that failed chemotherapy with cyclosporine expressed only MRP. One RB enucleated 2 years after failing chemotherapy with cyclosporine, despite radiation and salvage chemotherapy, expressed both p170 and MRP. Two metastatic RBs that expressed both p170 and MRP at diagnosis and at recurrence failed chemotherapy without cyclosporine, whereas one metastatic RB that expressed neither protein was cured by chemotherapy without cyclosporine. MRP may result in failure of chemotherapy despite the elimination of p170-expressing clones by cyclosporine.

Introduction

The 190-kDa MRP<sup>1</sup> confers a pattern of drug resistance <em>in vitro</em> that is similar to that of the multidrug resistance P-glycoprotein gene product, p170 (1–5). Because many studies have found that the presence of p170 is strongly associated with failure of chemotherapy in neuroblastoma, sarcoma, leukemia, lymphoma, and myeloma (6–10), trials that test whether cyclosporine or verapamil, inhibitors of p170 in <em>vitro</em>, can improve the results of chemotherapy (8–10) are ongoing. The role of MRP in the failure of chemotherapy remains poorly defined.

After myeloid leukemia patients had failed cyclosporine-modulated chemotherapy (8), p170 was reported to be undetectable, suggesting that the p170-expressing tumor cells had been eliminated. If failure of chemotherapy in these patients was due to resistance to cyclosporine, p170 should continue to be expressed. Alternative mechanisms of drug resistance, such as MRP, might account for failure of chemotherapy despite the use of inhibitors of p170.

MRP is a member of the same ATP-dependent transmembrane transporter superfamily as p170 (3, 5). Transfection of MRP cDNA confers a similar pattern of resistance to important cytotoxic anthracyclines, Vinca alkaloids, antibiotics, and epipodophyllotoxins (3–5). Furthermore, deletion of the MRP gene (16p13.1) on the inverted chromosome 16 of myelomonocytic leukemia correlated with a better outcome, suggesting that allelic loss of MRP might confer increased drug sensitivity (11). Because MRP is not blocked by the present inhibitors of p170 (1–5), it is important to establish whether MRP is the cause of failure to reverse multidrug resistance.

Until recently, chemotherapy rarely cured intraocular RB, which was often successfully treated by radiation, incurring a 30% risk of radiation-induced secondary cancers by 40 years in children with germ-line <em>RB1</em> gene mutations (12). We have found that p170 expression occurs more frequently in RB that failed therapy than in untreated RB (13), suggesting that overexpression of p170 could cause failure of chemotherapy in RB, as in the other previously reported cancers (6–10).

We have recently shown that chemotherapy with cyclosporine but without radiation had a high cure rate for intraocular RB: 91% of previously untreated tumors remained relapse-free, and 70% of those treated with chemotherapy and cyclosporine after having relapsed without cyclosporine were salvaged at a median follow-up of 2.8 years (14, 15). Even for those with the worst prognosis, eyes with RB that had seeded into the vitreous, the cure rate without radiation was 88%, much better than that previously reported with radiation (16), with the same chemotherapy without cyclosporine (17), or with the same chemotherapy without but with radiation (18). We now report that MRP expression is also associated with the rare failures of chemotherapy in RB despite the use of cyclosporine.

Patients and Methods

Patients. Since 1991, we have recruited 31 consecutive bilateral nonmetastatic RB patients, ages 15 days–5.3 years, into a carboplatin-vincristine-teniposide trial with cyclosporine but no radiation, approved by our research ethics board. The patient characteristics and protocols have been reported elsewhere (14, 15). Irreversibly damaged eyes were enucleated at diagnosis. We treated 40 eyes of 31 patients with chemotherapy with cyclosporine, consolidated with laser with/without cryotherapy. In the precyclosporine era (before 1991), we treated 19 nonmetastatic RB patients with vincristine-teniposide or vincristine-doxorubicin-cyclophosphamide protocols with/without radiation and treated 3 metastatic RB patients with radiation and vincristine-doxorubicin-cyclophosphamide or 8-in-1 protocols.

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3 The abbreviations used are: MRP, multidrug resistance protein; RB, retinoblastoma.
controls of these antibodies (I, 2). QCRL1 does not cross-react with p170, nor that have been quantified by immunoblot, including the sensitive and the 8-, clonal antibodies directed against separate p170 epitopes (C494, specific for controls. We stained the formalin-fixed samples three times with two mono treated with chemotherapy with/without cyclosporine. Eighteen samples from p170 or MRP in the primarily enucleated eye does not necessarily represent Results MRP-transfected HeLa lines that stained negative or 1+ to 3+ for MRP (3—7, and 50—100-fold resistant doxorubicin-selected small cell lung cancer or stained negative or 1+ to 5+ for p170 and the sensitive and the 2—3-, 4—12-, and 16-, 64-, 510-, 1000-fold resistant vincristine-selected ovarian cancer lines that stained negative or 1+ to 3+ for MRP (QCRL1 and QCRL3 are MRP-specific) and with isotype-matched negative controls of these antibodies (1, 2). QCRL1 does not cross-react with p170, nor does C219 cross-react with MRP (2). Two observers, masked to the identity of samples and to each other’s scores, interpreted the results; the final interpretation was based on the consensus score. We scored samples with no p170-positive or MRP-positive tumor cells as negative, grading those with p170-positive tumor cells from 1+ to 5+, and grading those with MRP-positive tumor cells from 1+ to 3+. We used appropriate controls with p170 and MRP that have been quantified by immunoblot, including the sensitive and the 8-, 16-, 64-, 510-, 1000-fold resistant vincristine-selected ovarian cancer lines that stained negative or 1+ to 5+ for p170 and the sensitive and the 2—3-, 4—12-, and 50—100-fold resistant doxorubicin-selected small cell lung cancer or MRP-transfected HeLa lines that stained negative or 1+ to 3+ for MRP (3—7, 19, 20).

Results Overall, we detected MRP in 12 of 30 samples (4 of 22 at diagnosis and 8 of 8 at relapse) and p170 in 13 of 32 samples (7 of 23 at diagnosis and 6 of 9 at relapse). There was considerable heterogeneity in the patterns and expression levels of p170 and MRP in different samples obtained at the same or different time points for each patient and between different patients (Fig. 1, A—H). All IgG control samples were negative. The observers were unanimous in interpreting whether or not each sample was positive for p170 or MRP, varying only in their scoring of the precise degree of positivity. We observed focal or diffuse p170 staining in the plasma membrane and Golgi, and MRP was observed in the plasma membrane and cytoplasmic endocytotic vesicles. The normal retina did not express p170 or MRP.

Both p170 and MRP were found more commonly at relapse than at diagnosis and found more often in metastatic than in nonmetastatic RB. For the 25 samples from 23 nonmetastatic RB patients, we found MRP in 7 of 7 samples at intraocular relapse but in only 1 of 18 samples at diagnosis and found p170 in 4 of 7 samples at intraocular relapse but in only 3 of 18 samples at diagnosis (Tables 1 and 2). However, for the three metastatic RB patients, we found both MRP and p170 present in two of two samples at relapse and in two of three samples at diagnosis.

In the precyclosporine era (before 1991), 22 eyes of 19 bilateral RB patients that did not require enucleation at diagnosis were treated with chemotherapy with/without cyclosporine. Thirty-two patients (of 18 newly diagnosed patients and 1 of 3 previously relapsed patients) at a median follow-up of 5.6 years (14) and thus could not be biopsied for p170 and MRP. Fifteen eyes failed therapy, 13 by 1 year and 2 by 2 years. Six of these failures occurred before 1991: three were salvaged by irradiation, but three were enucleated, all of which showed coexpression of MRP with p170 (Table 1). The nine eyes that failed after 1991 were recycled into the cyclosporine trial.

In the cyclosporine era (since 1991), we consolidated the chemotherapy-cyclosporine response with laser with/without cryotherapy in 40 eyes of 31 bilateral RB patients. At a median follow-up of 2.8 years, we achieved a 91% cure rate in 26 of 28 newly diagnosed RBs and a 70% cure rate in 9 of 12 relapsed intraocular RBs, 9 of which cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on July 16, 2017. © 1997 American Association for Cancer Research.
were previously treated by us (see above), and 3 of which were referred from elsewhere (14, 15). None of these 35 cured eyes were tested for p170 and MRP because they cannot be biopsied. Five eyes (two newly diagnosed eyes and three previously relapsed eyes) failed therapy despite cyclosporine, all by 1 year. One of these failures was salvaged with radiation, but four were enucleated (Table 2). All three eyes that were enucleated immediately after failing chemotherapy with cyclosporine expressed MRP (Fig. 1B) but not p170 (Fig. 1A). In one eye in which enucleation was delayed for 2 years while giving radiation and the non-p170 substrate topoisomerase I inhibitor topotecan as salvage chemotherapy, we detected high levels of MRP (Fig. 1D) with p170 (Fig. 1C). The expression of p170 or MRP in the primarily enucleated RB did not correlate with the chemotherapy response in the retained fellow eye that could not be biopsied before treatment (Tables 1 and 2).

Discussion

The present series is small because: (a) RB is a rare disease occurring only in 1 of 20,000 live births; (b) extraocular RB is even rarer (less than 10% of all cases); (c) less than 40% of intraocular RB tumors are treated with chemotherapy; and (d) very few patients fail our present cyclosporine regimen to require eye enucleation, allowing assessment of the drug resistance proteins. The fact that there are so few eyes available for study is a triumph for the children, but this makes a large scientific series impossible.

Immunohistochemistry is a standard method to score for MRP or p170 (21), because it allows single-cell detection of clinically relevant levels of p170 or MRP (6, 7, 21). This is particularly applicable to RB (Table 3). Two children died of metastatic RB despite radiation and intensive chemotherapy without cyclosporine. We found that before treatment, these two RBs coexpressed p170 (Fig. 1E) and MRP (data not shown) in both the eye and the metastasis (not shown), with even stronger expression at relapse (Fig. 1F). One patient with bone marrow and orbital metastasis in which RB both p170 (Fig. 1G) and MRP (Fig. 1H) were undetectable at diagnosis is an 18-year survivor after chemotherapy and radiation.
sarily reflect the protein levels (4, 20). Except for the high-expression carcinomas (22), MRP and p170 levels in tumors are generally lower than in gene-amplified, drug-selected cell lines (3, 5, 22). Northern blot, RT-PCR, and immunoblot measure tumor cells pooled with normal stroma and hematopoietic cells that might or might not show MRP or p170 expression that has been reported to occur widely in normal tissues. Some of these techniques might not be sensitive enough to detect the low and heterogeneous MRP or p170 expression in patient samples that is clinically important. Furthermore, the very limited RB sample size and heterogeneity of p170 or MRP expression preclude anything but in situ studies.

We have previously hypothesized that the poor cure rate of RB by chemotherapy without radiation may be due to drug resistance conferred by p170 (13). To show that p170 limits the response to chemotherapy, it is necessary to find p170 in unresponsive but not in responsive tumors, predict the outcome of chemotherapy by prospective testing, and salvage patients with resistant tumors by using inhibitors of p170. In many types of tumors, the presence of p170 before treatment is strongly associated with poor outcome (6–10). Similar correlation is impossible to establish for intraocular RB, because these tumors cannot be safely biopsied before treatment. However, the finding of p170 in all of the unresponsive tumors but in only a fraction of untreated tumors suggests the possibility that p170 might be responsible for the resistance to chemotherapy.

Because we postulated that p170 might be rate-limiting for the response of RB to chemotherapy (13), we added the p170 inhibitor cyclosporine to our chemotherapy protocol. Our greater than 90% success rate in newly diagnosed RB and 70% success rate in previously treated but relapsed RB suggest that cyclosporine does enhance the efficacy of chemotherapy (14, 15). Higher doses of cyclosporine, higher blood levels, and the greatest projected tissue exposure correlated with the best outcome (14, 15). Notably, poor-prognosis RB with vitreous seeding did better with chemotherapy and cyclosporine than it did with radiation (16), with the same chemotherapy without cyclosporine (17), or with the same chemotherapy without cyclosporine but with radiation at other centers (18). Despite significant improvement of the long-term efficacy of chemotherapy with cyclosporine (14, 15), we cannot conclusively prove that the cyclosporine acts by modulating p170, because we cannot test intraocular RB tumors before treatment.

Whether MRP is also a clinically relevant cause of failure of chemotherapy has not been firmly established. The expression of MRP before treatment has been shown to significantly correlate with the outcome of treatment in neuroblastoma (23). We show here that MRP was coexpressed with p170 in two RB patients that were metastatic at diagnosis and later died (Table 3) and in three of three intraocular RB patients enucleated immediately after failure of chemotherapy without cyclosporine (Table 1). These observations suggest but do not prove that both of these drug resistance proteins might have contributed to therapeutic failure. The three of three RBs that were enucleated immediately after failing chemotherapy with cyclosporine expressed MRP but not p170 (Table 2), suggesting that despite possible eradication of the p170-positive clones with cyclosporine, therapy conceivably might have still failed because of the occurrence of MRP, which is poorly or not at all blocked by cyclosporine (5, 24). Furthermore, the one eye enucleated 2 years after having failed chemotherapy with cyclosporine despite additional radiation and to-potecan showed high levels of both MRP and p170. Both a cyclosporine-resistant MRP clone and a reemergent p170-positive clone might possibly be responsible for the failure of salvage therapy in this child.

Most reported trials that have used 24-h infusions of cyclosporine for up to 6 days have shown modest efficacy but considerable toxicity from increased systemic exposure to the cytotoxins (8, 9), caused by cyclosporine alteration of drug metabolism or prolonged inhibition of the p170 drug-efflux pump in p170-expressing normal tissues such as the kidney, liver, bowel, blood-brain barrier, and myeloid progenitors. Conversely, we have administered short 3-h infusions of cyclosporine on two sequential chemotherapy days with acceptable chemotoxicity and excellent dose intensity (14, 15). Consequently, the efficacy we have observed is unlikely to be due entirely to the increased systemic cytotoxic drug exposure from cyclosporine (14, 15). However, because we have not tested the pharmacokinetics of the cytotoxins, we cannot exclude the possibility that cyclosporine may have caused a small but important increase in the true dose intensity, without noticeably increasing the overall chemotoxicity. Alternatively, high concentrations of cyclosporine might have persisted in the eye sanctuary to effectively inhibit p170 locally, a mechanism that is not available to other types of tumors. Cyclosporine might also have modulated non-p170 mechanisms of resistance, such as enhancing the responsiveness to epipodophyllotoxins even in sensitive cells (25) or suppressing the carboxiplatin-induced expression of c-fos, c-myc, or other genes required for repair of drug-induced DNA damage (26).

We were able to biopsy metastatic RB before treatment. The two RBs that expressed both p170 and MRP in the intraocular and metastatic tumors before treatment were not cured by therapy and succumbed. The p170-negative and MRP-negative metastatic RB tumor in one patient was cured by chemotherapy. This very rare survival of metastatic RB might be due to the absence of expression of the two drug resistance proteins.

MRP is widely expressed in normal tissues, but its physiological function is unknown (3), except possibly as a transporter for the endogenous glutathione conjugate leukotriene C4 and the conjugated estrogen 17β-estradiol glucuronide and xenobiotics (5). Precisely how MRP causes multidrug resistance is uncertain, but in addition to pressing the carboplatin-induced expression of c-los, c-myc, or other genes, it readily blocked by the known inhibitors of p170 (3—5), it will be necessary to develop blockers specific for MRP to solve the very important clinical problem of multidrug resistance. Although RB is a rare tumor in which failures are rare with our present chemotherapy with cyclosporine, it may serve as a model to gain useful information important for the more common tumors.

References

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