Expression of the Multidrug Resistance-associated Protein (MRP) Gene Correlates with Amplification and Overexpression of the N-myc Oncogene in Childhood Neuroblastoma

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Abstract

The MRP gene (Cole et al., Science (Washington DC), 258: 1650–1654, 1992) encodes a membrane-bound glycoprotein the expression of which correlates with non-P-glycoprotein-mediated multidrug resistance in a variety of cultured human cell lines. Using an RNA-polymerase chain reaction assay, expression of this gene was examined in the highly chemoresistant pediatric malignancy, neuroblastoma. MRP expression was observed in 5 human neuroblastoma cell lines and in all 25 primary neuroblastoma tumors of stage I through IVS. Tumors with amplification of the N-myc oncogene were found to have significantly higher MRP expression than those with no amplification (P = 0.0016). Expression of the MRP gene in the tumor specimens was highly correlated with expression of the N-myc gene (P = 0.0009), while expression of the MDR1 gene, encoding P-glycoprotein, was not related to expression of either the N-myc or MRP genes. Decreased expression of the N-myc oncogene in neuroblastoma cell lines SH-SYSY and BE(2)-C, following treatment with retinoic acid, was paralleled by down-regulation of MRP gene expression, contrasting with increased expression of the MDR1 gene. Expression of the MRP gene is thus common in both primary neuroblastoma tumors and cultured cell lines, and correlates with amplification and overexpression of the N-myc oncogene, which is central to the malignant phenotype of this disease.

Introduction

Neuroblastoma, a disease which arises from sympathetic nervous tissue, is the most common solid tumor of early childhood. Although the advent of combination chemotherapy has led to significant improvements in the survival rates for most forms of childhood cancer, neuroblastoma remains a chemoresistant disease. This is particularly the case for patients whose tumors exhibit amplification of the N-myc oncogene, which is a powerful indicator of both response to chemotherapy and poor outcome (1). Such patients are characterized refractory, even to intensive combination chemotherapy regimens, and have a dismal prognosis, with progression-free survival rates of less than 10% (2). The simultaneous resistance to a diverse range of structurally and functionally unrelated drugs, as exemplified by aggressive neuroblastomas, is referred to as MDR3 (3). The phenomenon of MDR has been well described in the literature and is commonly associated with increased expression of P-glycoprotein encoded by the MDR1 gene (3). The role of the MDR1 gene in mediating MDR in neuroblastoma is unclear, however, and the mechanisms underlying the chemoresistant phenotype of this tumor are poorly understood (4). The recently described multidrug resistance-associated protein (MRP) gene, identified by Cole et al. (5), encodes a novel membrane transport protein, the overexpression of which has been associated with a number of non-P-glycoprotein-mediated MDR phenotypes in vitro. Only a single published report has described MRP expression in tumor samples from patients with hematopoietic malignancies (6) and as yet, there are no published reports on the expression of MRP in human solid tumor samples. Hence, the role of MRP in clinical drug resistance remains to be elucidated (7). We have therefore used a competitive RNA-PCR assay to examine MRP expression in neuroblastoma tumors and cell lines. The data indicate that MRP expression correlates with amplification and overexpression of the N-myc oncogene, suggesting a role for MRP in the malignant, chemoresistant phenotype of this disease.

Materials and Methods

Patient Samples. The 25 neuroblastoma tumor samples used in this study were either accrued from patients presenting at the Prince of Wales Children’s Hospital (Sydney, Australia) or were obtained from the POG Neuroblastoma Tumor Bank. Samples from the POG Neuroblastoma Tumor Bank were sent to the investigators for analysis after the proposed research project was reviewed and approved by the POG Neuroblastoma Subcommittee. At least 3 tumors representing each clinical disease stage (i.e., stages I, II, III, IV, and IVS) were included in the study. For all samples, the numbers of copies of the N-myc oncogene per haploid genome had been independently determined by Southern analysis prior to this study, and five of the tumors (three stage III and two stage IV) had been found to display N-myc gene amplification, with copy numbers ranging from 8 to 300.

Cell Lines. IMR-32 cells were obtained from the American Type Culture Collection (Rockville, MD), SH-SYSY and BE(2)-C cell lines were generously supplied by Dr. J. Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY), and LA-N-1 and LA-N-2 were kindly provided by Dr. R. Seeger (Los Angeles Children’s Hospital, Los Angeles, CA). All lines were maintained as monolayer cultures at 37°C in RPMI 1640 supplemented with 10% fetal calf serum. In vitro treatment with all-trans-RA (Sigma Chemical Co.) involved continuous exposure of SH-SYSY and BE(2)-C cell lines to RA (10 μM) or ethanol vehicle for 2, 6, or 9 days.

RNA Analysis by Polymerase Chain Reaction (RNA-PCR). Total cytoplasmic RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase and random hexanucleotide primers, essentially as described by Noonan et al. (8). A cDNA amount representing 50 ng of RNA was subjected to PCR for 35 cycles in a final volume of 25 μl using 1 unit of AmpliTaq Polymerase (Perkin Elmer Cetus). Following an initial denaturation of 3 min at 94°C, each cycle consisted of 45 sec at 94°C, 45 sec at 55°C, and 90 sec at 72°C. Target (MRP, N-myc, or MDR1) and control (β2-microglobulin or GAPDH) gene sequences were amplified in the same reaction, following the use of gene-specific oligonucleotide primers: MRP forward primer, 5'-TTCTCCTCCACATGACCAGG-3'; MRP reverse primer, 5'-CCAGGAATATGCCCCGACCTC-3'; N-myc forward primer, 5'-CGACCAAAAGCCCTCTAGTA-3'; N-myc reverse primer, 5'-CAGCTTGGTGTTGGAGGAGG-3'; MDR1 forward primer, 5'-CCCATTGGCAATAGCAG-3'.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: MDR, multidrug resistance; MRP, multidrug resistance-associated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; cDNA, complementary DNA; POG, Pediatric Oncology Group.
The level of MRP gene expression in three of these cell lines was determined, specifically in MCF7/VP (Lane 7). The samples in Lanes 5—8 have been previously reported to have moderate levels of MRP gene expression (5, 6), while high-level expression was evident in the MCF7/VP cell line (Fig. 1A, Lane 7), in which the MRP gene is amplified. Several samples of normal peripheral blood mononuclear cells, previously reported to have moderate levels of MRP gene expression (5, 6), were all found to express MRP at levels intermediate between those of human liver and the MCF7/VP cell line (representative samples are shown in Fig. 1A, Lanes 2 and 3). In comparison with these reference standards, high-level expression of the MRP gene was apparent, as indicated in a representative gel (Fig. 1B) which includes tumors of all stages, both with (Fig. 1B, Lanes 5–8) and without (Fig. 1B, Lanes 1–4) N-myc gene amplification.

MRP expression was examined in specimens of primary untreated neuroblastoma. A panel of 25 tumors, including at least 3 samples from each clinical stage of the disease, was evaluated, and the MRP gene was found to be expressed in all 25 tumors analyzed, regardless of clinical stage. Variation between tumors in the level of MRP expression was apparent, as indicated in a representative gel (Fig. 1B) which includes tumors of all stages, both with (Fig. 1B, Lanes 5–8) and without (Fig. 1B, Lanes 1–4) N-myc gene amplification. Following densitometric analysis, the level of MRP expression in each tumor was quantitated relative to that of β2-microglobulin by comparing the ratio between the target and control PCR products for each sample. A similar analysis was also performed on each tumor for expression of the N-myc oncogene and the MDR1 gene. The mean level of MRP expression in the 25 neuroblastoma samples (mean PCR ratio, 0.744 ± 0.058) was significantly greater than the mean expression of this gene in the peripheral blood mononuclear samples obtained from healthy volunteers (mean PCR ratio, 0.526 ± 0.077; P < 0.05), although significantly lower than the level of MRP expression in untreated neuroblastoma.

Analysis of MRP Gene Expression in Human Neuroblastoma Cell Lines and Primary Tumors. In an initial study, MRP gene expression was determined in a number of human neuroblastoma cell lines. The 140-base pair MRP PCR product, anticipated on the basis of published sequence (5), was amplified in all five cell lines examined, namely, SH-SY5Y, LA-N-1, LA-N-2, IMR-32, and BE(2)-C. The level of MRP gene expression in three of these cell lines was evaluated using a competitive PCR reaction involving coamplification of the MRP gene with a control gene, β2-microglobulin. We have demonstrated previously the utility of this assay for semi-quantitative analysis of gene expression in neuroblastoma tumors and cell lines, using either β2-microglobulin or GAPDH as control gene sequences (10). Both of these control genes are expressed at consistent levels in primary neuroblastoma tumors, regardless of clinical stage (10), and have been used as control genes in a number of studies of gene expression in primary tumor material, including both neuroblastoma and other malignancies, and using both PCR-based and Northern analyses (14, 15). For comparative purposes, samples known to have low, intermediate, and high levels of MRP gene expression were included in the analysis. Consistent with previous reports (6, 12), normal human liver was found to express low levels of the MRP gene (Fig. 1A, Lane 1), while high-level expression was evident in the MCF7/VP cell line (Fig. 1A, Lane 7), in which the MRP gene is amplified. Several samples of normal peripheral blood mononuclear cells, previously reported to have moderate levels of MRP gene expression (5, 6), were all found to express MRP at levels intermediate between those of human liver and the MCF7/VP cell line.

Southern Analysis of the MRP Gene. DNA was isolated from the tumor samples and cell lines by phenol extraction prior to EcoRI digestion and Southern hybridization as described previously (13). The PCR product amplified by the MRP gene-specific primers described above was purified from photographic negatives (9, 10) and a ratio between the target and control PCR products was determined. Following PCR, aliquots (10 µl) were subjected to electrophoresis on 12%

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* E. Schneider, personal communication.
MRP expression in childhood neuroblastoma

Fig. 3. Correlation between expression of the N-myc and MRP genes in 25 primary neuroblastoma tumors. Following independent, competitive RNA-PCR analyses using primers for either the N-myc or MRP genes, together with control β2-microglobulin gene primers, ratios between target and control gene products were determined densitometrically for each sample. Linear regression analysis relating the PCR ratio for N-myc expression to that of the MRP gene for each sample indicated a highly significant relationship. •, tumors with N-myc gene amplification; ○, tumors without N-myc gene amplification.

Discussion

Direct evidence for involvement of the MRP gene in mediating drug resistance has recently been provided with the demonstration that HeLa cells, transfected with MRP expression vectors, exhibited increased resistance to a number of MDR drugs including doxorubicin, vincristine, and VP-16 (18). The finding that MRP is capable of conferring an MDR phenotype has since been confirmed by expression cDNA library transfer (19). To date, the role of MRP has been limited to studies of cultured cell lines exhibiting non-P-glycoprotein amplification and overexpression of the N-myc gene and expression of the MRP gene, the possibility of MRP gene amplification in tumors expressing high MRP levels was investigated. Genomic DNA was isolated from 15 of the tumors, including the 2 with the highest levels of MRP gene expression, both of which carried multiple copies of the N-myc oncogene. Following Southern analysis, no increase in MRP gene copy number was detected in any of the tumors (data not shown).

Modulation of Gene Expression following RA-induced Differentiation of Neuroblastoma Cell Lines. To further investigate the relationship between expression of the N-myc and MRP genes, neuroblastoma cell lines SH-SY5Y and BE(2)-C were exposed in vitro to the differentiating agent, RA, which has previously been shown to induce down-regulation of the N-myc oncogene (16). In the present study, continuous RA treatment resulted in growth arrest and neurite extension in both cell lines (data not shown). Competitive RNA-PCR analysis was performed to determine expression of the MRP, N-myc, and MDR1 genes following exposure of cells to RA for various lengths of time. As anticipated, there was a highly significant decrease over time (P < 0.0001) in the level of N-myc expression following RA treatment (Fig. 4). There was no significant difference in the effect of RA treatment on the SH-SY5Y (Fig. 4, top) and BE(2)-C (Fig. 4, bottom) cell lines (P > 0.05 for the interaction between cell lines and repeated measures effects). Concomitant with the RA-induced down-regulation of the N-myc gene, MRP gene expression was also observed to decrease significantly (P = 0.0017) following RA treatment, and again, no difference in the effect was observed (P > 0.05) between the two cell lines (Fig. 4). Consistent with previous reports (17), both cell lines demonstrated increased expression of the MDR1 gene (P < 0.0005) following exposure to RA (Fig. 4).
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Among the primary tumor specimens examined, only a single tumor without N-myc gene amplification displayed MRP levels above the median level observed in the N-myc-amplified tumors. Interestingly, this tumor was from a stage IV patient who, despite favorable molecular indicators (nonamplified N-myc and high trk expression), died shortly following diagnosis from rapidly progressive disease. This finding of high-level MRP expression in a tumor without N-myc gene amplification is similar to the result observed with the SH-SYSY neuroblastoma cell line, which despite having only a single copy and concomitant low-level expression of the N-myc oncogene, nevertheless expressed high levels of MRP. Since expression of the MRP gene in the absence of N-myc gene amplification can clearly be induced by exposure of cells to cytotoxic drugs (5, 7, 11, 12), the high level of MRP gene expression present in the SH-SYSY cells may have been induced by prior exposure of the donor patient to combination chemotherapy which included a number of MRP-associated drugs. In this context, it is of interest to note that the high level of MRP expression in the SH-SYSY cells is consistent with both the clinical resistance of these cells to chemotherapy and their relatively high level of resistance in vitro to MRP-associated antimicrobial agents such as vincristine and colchicine (26).

In view of the studies demonstrating that increased expression of the MRP gene confers a multidrug resistance phenotype (18, 19), the relationship demonstrated in this study between increased MRP gene expression and both amplification and overexpression of the N-myc oncogene suggests a potential link between the molecular mechanisms mediating the malignant and chemoresistant phenotypes of neuroblastoma. Thus, N-myc may modulate MRP gene expression in neuroblastoma, which in turn may contribute to the chemoresistance displayed in vivo by tumor cells with N-myc gene amplification. Further studies comparing levels of MRP expression in neuroblastoma tumors before and after treatment with cytotoxic drugs and correlating MRP expression with clinical outcome will be required to clarify the association between expression of the MRP gene and multidrug resistance in human neuroblastoma tumors. Moreover, understanding the precise role of the MRP gene in neuroblastoma must await clarification of the function of the MRP gene product in normal cells. Nevertheless, the present finding of increased MRP expression in aggressive primary human neuroblastoma tumors implicates a clinically relevant role for expression of the MRP gene.

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References


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