Intravitreal Daunomycin Induces Multidrug Resistance in Proliferative Vitreoretinopathy

Peter Esser,1 Daniel Tervooren,1 Klaus Heimann,1 Norbert Kociok,1 Karl Ulrich Bartz-Schmidt,1 Peter Walter,1 and Michael Weller2

PURPOSE. Adjuvant intravitreal daunomycin is frequently used for the management of proliferative vitreoretinopathy (PVR). In this study the authors examined whether daunomycin could induce multidrug resistance (MDR), mediated by the mdr-1 gene product P-glycoprotein, in the cells responsible for reproliferation in vivo and in human retinal pigment epithelial (RPE) cells in vitro.

METHODS. Expression of P-glycoprotein was examined by immunohistochemistry in surgically removed epiretinal membranes. The cellular source of P-glycoprotein was examined by colabeling for cytokeratin, glial fibrillary acidic protein, and the macrophage marker EBM-11. P-glycoprotein expression by cultured RPE cells was assessed by reverse transcription-polymerase chain reaction and immunoblot analysis. Daunomycin toxicity was quantified by crystal violet assay.

RESULTS. P-glycoprotein expression was detected in 10 of 10 patients pre-exposed to intravitreal daunomycin. In contrast, epiretinal membranes from only 2 of 13 patients never exposed to daunomycin showed faint P-glycoprotein expression. P-glycoprotein expression was strong within 8 months after daunomycin treatment and faded thereafter. Colocalization studies demonstrated predominant expression of P-glycoprotein by RPE cells. Pre-exposure of cultured human RPE cells to subtoxic concentrations of daunomycin induced resistance to daunomycin that was sensitive to the MDR inhibitor, verapamil. Induction of the MDR phenotype in RPE cells by daunomycin was associated with a minor increase in the mdr-1 mRNA level but a prominent increase in P-glycoprotein expression, thus suggesting a primarily translational mechanism of MDR development in human RPE cells.

CONCLUSIONS. Intravitreal daunomycin induced P-glycoprotein expression in PVR. Reproliferation in daunomycin-pretreated patients probably necessitates cotreatment with daunomycin and inhibitors of multidrug resistance such as verapamil or administration of antiproliferative drugs such as 5-fluorouracil, which act in a MDR-independent fashion. (Invest Ophthalmiol Vis Sci. 1998;39:164-170)

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Proliferative vitreoretinopathy (PVR) is a common cause of blindness in Western societies. Vitrectomy with membrane peeling using either gas or silicone oil tamponade is the treatment of choice. Because reproliferation after vitrectomy is common, adjuvant intravitreal pharmacotherapy using antiproliferative agents such as daunomycin and 5-fluorouracil has been recommended.1-9 The management of recurrent PVR after such treatment is difficult and often unsuccessful. The role of adjuvant pharmacotherapy in recurrent PVR is not well-defined.

The development of resistance to multiple drugs is a common problem in cancer chemotherapy. Drug resistance is often mediated by the human multidrug resistance mdr-1 gene product, P-glycoprotein. This protein confers resistance to a variety of drugs including vinca alkaloids and anthracyclins such as daunomycin, colchicine, and taxol in neoplastic and non-neoplastic cells.9,10 P-glycoprotein acts as an energy-dependent drug efflux pump to maintain low intracellular levels of cytotoxic drugs.11-14 Exposure to several drugs induces activation of the mdr-1 gene and development of a multidrug resistant (MDR) phenotype.

Here we investigated whether intravitreal daunomycin, administered for the prevention of reproliferation in PVR, induces P-glycoprotein expression in vivo and whether daunomycin exposure of cultured retinal pigment epithelial (RPE) cells, which play a central role in PVR,15 induces a MDR phenotype in these cells in vitro.

MATERIALS AND METHODS

Immunohistochemistry

The general immunostaining procedures were performed as described previously.16 Epiretinal membranes were obtained from 23 patients undergoing vitrectomy for traumatic PVR and PVR after rhegmatogenous retinal detachment (idiopathic PVR). All patients provided informed consent before surgery. The decision for the use of daunomycin in 10 of these patients had been based on medical reasons only (severity of disease, proprietary interest category: none.

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TABLE 1. P-glycoprotein Expression in Recurrent Proliferative Vitreoretinopathy

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The table indicates, in this order, case identification (A, no prior daunomycin treatment; B, prior daunomycin treatment; C and D, membranes from same patient and subsequent operations), type of proliferative vitreoretinopathy (PVR) (tr, traumatic; id, idiopathic, this is PVR after rhegmatogenous retinal detachment), number of our case registry (No), age, gender, interval to last surgical intervention, stage of PVR (A, anterior; P, posterior; 1-4 numbers of quadrants involved corresponding to grade C of the nomenclature introduced by Machemer et al), prior daunomycin treatment (Dauno, + or −), intraocular foreign body (IOFB), vitreous replacement by silicone oil (S) or long acting gas (C3F8, G), cellularity of membrane (number of cells/low power field), intensity of P-glycoprotein (Pgp) staining (−, no staining; (+), <10%; +, 10-30%; + +, >30% of cells labeled), GFAP staining, cytokeratin staining (Cyt), and macrophage staining (Mc) (percentage of positive cells and results of colocalization studies with P-glycoprotein (Pgp) (− or +)).

multiple cells in the vitreous cavity, fulminant progression of PVR. The membranes were immediately frozen at −70°C and cut on a cryostat for a minimum of 10 minutes. In this retrospective study, the incubation of the epiretinal membranes with human monoclonal P-glycoprotein antibody (Signet, Dedham, MA) was followed by labeling with alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Dakopatts; Glostrup, Denmark) and visualization by Fast Red substrate (Dakopatts). Immunoreactivity for P-glycoprotein was graded as outlined in Table 1. Immunochemical identification of RPE cells with cytokeratin antibodies (Dakopatts), glial cells with glial fibrillary acidic protein antibodies (Dakopatts), and macrophages with the EBM-11 antibody (Dakopatts) was performed accordingly. Antibodies were diluted in phosphate-buffered saline containing 0.5% bovine serum albumin. Negative controls were performed by substituting the primary antibody with nonimmune mouse immunoglobulin G (Sigma Chemical, St. Louis, MO).

Cell Culture and Cytotoxicity Assays

Human RPE cells were prepared from keratoplasmy donor eyes and cultured in minimal essential medium (D-VaL; Sigma) containing 15% fetal calf serum as previously described.17 Second passage cells were used for the cytotoxicity experiments. Briefly, 2 × 10^4 human RPE cells were adhered to 96-well plates for 24 hours and subsequently exposed to daunomycin (0.01-100 μM) for 10 minutes. The cells were rinsed twice, and fresh medium containing 5% fetal calf serum was added. Survival was assessed by crystal violet staining 24 hours later. Verapamil, an inhibitor of P-glycoprotein activity, was added to the medium at a final concentration of 4 μM in some experiments. A MDR phenotype was induced by maintaining the RPE cells in medium containing subtoxic concentrations of daunomycin (100-200 nM) for 2 weeks or by exposing the cells to a clinically used concentration of daunomycin (13 μM) for 10 minutes.

RNA Preparation Using Reverse Transcription–Polymerase Chain Reaction

Total RNA was prepared by guanidium–phenol extraction from untreated cultured human RPE cells from freshly harvested cells from passage P0 and from untreated and daunomycin-treated (100–200 nM) human RPE cells from passage P2 using TRI Reagent (Sigma) according to the protocol of the manufacturer. The reverse transcription (RT) reaction was performed on 500 ng of total mRNA using the a preamplification system for first-strand cDNA synthesis (SUPERSCRIPT; Life Technologies, Eggenstein, Germany) with oligo(dT)12-18 primers. Amplification of a 745bp mdr-1 cDNA fragment was performed using the primers HMDR1UP (5'AAATATCAGTGCACGCCACATC-3') and HMDR2DO (5'TGCCCATTCTGAACACCACTA-3'), chosen from the human mdr-1 mRNA se-
sequence (accession number: M14758). The primers were selected from exons 24 and 28 using primer analysis software (OLIGO 4.1; National Biosciences, Plymouth, MN), precluding amplification of contaminating genomic DNA. The cDNA was diluted with TE buffer (10 mM Tris-HCl, pH 8, 1 mM ethylenediaminetetraacetic acid) corresponding to RT transcripts of 62.5, 12.5, 2.5, and 0.5 ng of total RNA. The diluted cDNA samples were mixed with 45 µl of polymerase chain reaction (PCR) mixture. The final reaction sample contained 50 mM Tris-HCl (pH 9.0), 15 mM (NH₄)₂SO₄, 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 µM of each primer, and 1 U of DNA polymerase from *Thermus brockianus* (PrimeZyme LE; Biometra, Göttingen, Germany). Thermocycling was performed with a thermocycler (PTC-100; MJ Research, Watertown, MA), using a hot start (heating of the mixture without DNA polymerase to 98°C for 5 minutes and addition of polymerase at 80°C) and 30 amplification cycles of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, followed by an incubation at 72°C for 10 minutes. Amplification products were separated on a 2% agarose gel, visualized with 1 µg/ml ethidium bromide, and documented on type 667 Polaroid print films.

**Immunoblot Analysis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Multiphor II electrophoresis unit; Pharmacia, Uppsala, Sweden) with subsequent blotting after protein transfer to nitrocellulose was performed as previously described.¹⁸ Cell lysates were adjusted to equal protein content (40 µg/lane). Proteins were separated on polyacrylamide gels. Immunoreactive P-glycoprotein was detected using a specific monoclonal antibody, followed by addition of horseradish peroxidase-conjugated anti-mouse immunoglobulin G. Chemiluminescence signals were obtained using the a kit (ECL Kit, RPN 2106; Amersham, Braunschweig, Germany).

**RESULTS**

**Adjuvant Intravitreal Daunomycin Induced P-glycoprotein Expression in Epiretinal Proliferative Vitreoretinopathy Membranes in Vivo**

To investigate a possible role of the MDR phenomenon in response to therapy in recurrent PVR, we examined samples of
23 PVR patients undergoing vitrectomy for reproliferation. Ten of these patients had received adjuvant intravitreal daunomycin after vitrectomy according to our standard protocol.5 Surgically removed epiretinal membranes of these patients were assessed for P-glycoprotein expression by immunocytochemistry (Fig. 1 and Table 1). Different amounts of P-glycoprotein were detected in 10 of 10 membranes from patients pretreated with daunomycin, whereas only 2 of 13 specimens from patients never exposed to intravitreal daunomycin showed faint P-glycoprotein immunoreactivity (Fisher exact test, P < 0.001).

The degree of immunoreactivity correlated with the interval between daunomycin exposure in that most prominent immunoreactivity was found with intervals between daunomycin exposure and reoperation of less than 8 months (Table 1). We did not detect any relationship between the age of the patients or history concerning type of PVR, ocular surgery, type of tamponade, and staining pattern of P-glycoprotein. In two cases, membranes were available from two subsequent operations on the same eye. P-glycoprotein immunoreactivity remained negative in one patient who had not received intravitreal daunomycin during the first vitrectomy (Table 1C). In contrast, a patient who had received daunomycin with the first operation (Fig. 1A) showed prominent P-glycoprotein immunoreactivity of a PVR membrane removed 2 months later (Table 1D and Figs. 1B, 1C).

Next we attempted to determine which cells in the PVR membranes were responsible for P-glycoprotein expression. As previously reported,16,17 we identified RPE cells, glial cells, and monocytes-macrophages by immunoreactivity for cytokeratin, glial fibrillary acidic protein, or the macrophage marker EBM-11. In the two patients without prior daunomycin exposure and faint P-glycoprotein immunoreactivity, P-glycoprotein-positive cells were cytokeratin-positive and thus presumably of RPE cell origin. Overall, RPE cells were the dominant cell type and responsible for most of the P-glycoprotein expression in the P-glycoprotein-positive membranes of daunomycin-pre-treated patients. P-glycoprotein-positive glial cells were detected in a single specimen (Table 1, B6). P-glycoprotein-positive monocytes-macrophages were present in membranes of 6 of 10 daunomycin-pre-treated patients. In some cases (B4, B7, B8), the cellular origin of P-glycoprotein-expressing cells could not be identified; they were negative for cytokeratin, glial fibrillary acidic protein, and EBM-11 in parallel sections.

Pre-exposure to Daunomycin Induced Verapamil-Sensitive, Multidrug Resistance-Type Resistance of Retinal Pigment Epithelial Cells to Daunomycin

To confirm daunomycin-mediated induction of the MDR phenotype in vitro, we examined cultures of human RPE cells. RPE cells are thought to play an important role in PVR and were identified by cytokeratin labeling as the major population in the surgical PVR specimens examined here (Fig. 1 and Table 1). A brief, 10-minute exposure of naive RPE cells to daunomycin resulted in concentration-dependent cytotoxicity and growth inhibition with an EC50 level of 13.2 ± 0.57 μM (Fig. 2). There was no significant EC50 shift for daunomycin when naive RPE cells were treated with daunomycin in the presence of verapamil (13.2 ± 0.57 μM compared with 12.1 ± 0.34 μM, n = 3, P > 0.05, Student’s t-test), a drug previously shown to reverse the MDR phenotype.20,21 Thus, the constitutive sensitivity of RPE cells to daunomycin appears not to be determined by constitutive P-glycoprotein expression. Next we asked whether prolonged exposure of the RPE cells to subtoxic concentrations to daunomycin enhanced their resistance to daunomycin. When the cells were pretreated with daunomycin at 100 nM for 2 weeks and subsequently treated with increasing toxic concentrations of daunomycin, the EC50 level
for daunomycin-induced cytotoxicity and growth inhibition increased from 13.2 μM to 26.8 ± 1.32 μM. Thus, pre-exposure to daunomycin induced significant resistance to a subsequent re-exposure to daunomycin (P < 0.01, Student’s t-test). The induction of resistance was abrogated by verapamil (EC<sub>50</sub>

= 14.9 ± 0.46 μM), suggesting that activation of mdr-1 gene expression was the underlying mechanism.

This was confirmed by RT-PCR and immunoblot analysis for mdr-1 gene and P-glycoprotein expression in daunomycin-treated RPE cells (Fig. 3). A 745-bp fragment of the mdr-1 cDNA was detected both in untreated P0 and P2 as well as in daunomycin-treated P2 cells. Serial dilutions revealed a moderate increase of mRNA levels in daunomycin-treated RPE cells (Fig. 3A). The detection of mdr-1 mRNA in naive RPE cells was difficult to reconcile with the observation that verapamil failed to alter daunomycin toxicity in RPE cells that had not been pre-exposed to daunomycin (Fig. 2), unless P-glycoprotein expression was significantly regulated on the translational rather than transcriptional level. Immunoblot analysis confirmed that this was indeed the case. Despite the detection of mdr-1 mRNA by reverse transcription-PCR (Fig. 3A), neither P0 nor untreated P2 RPE cells expressed the P-glycoprotein (Fig. 3B). In contrast, there was a strong signal after daunomycin exposure, suggesting that daunomycin-induced development of the MDR phenotype results mainly from enhanced mdr-1 mRNA translation or enhanced P-glycoprotein stability, or both.

Because in vivo RPE would only be exposed to daunomycin for a significantly shorter time period, we also investigated the upregulation of P-glycoprotein expression in cultured RPE cells after treatment in a manner presently in clinical use (13 μM, 10 minutes). We were able to demonstrate a slight up-regulation of P-glycoprotein expression 24 hours after treatment (lane 1). This increase in immunoreactivity was more pronounced after 3 days (lane 2) and diminished only slightly after 14 days after the initial treatment.

**DISCUSSION**

MDR is the phenomenon of cross-resistance to a wide variety of structurally and pharmacologically unrelated compounds in cells selected for resistance against a single drug. MDR is associated with decreased intracellular concentrations of cytotoxic drugs, which are kept low by an energy-dependent pump mechanism, involving P-glycoprotein, the product of the mdr-1 gene. Transfer of the mdr-1 gene into drug-sensitive cells confers the complete MDR phenotype. P-glycoprotein is
found at substantial levels in normal secretory cells of colon, kidney, liver, adrenal gland, and endothelial cells of the blood-brain barrier, indicating a physiological secretory function of the protein. We are not aware of a study on the expression of P-glycoprotein in the eye.

Reversal of MDR has become an important issue in cancer chemotherapy. PVR is characterized by the uncontrolled growth of non-neoplastic cells at the vitreoretinal interface. Removal of epiretinal membranes by vitrectomy alone results in short-term improvement, but reproliferation is frequent. Intravitreal application of daunomycin not only effectively controls experimental PVR but also improves clinical outcome in human patients. Yet, renewed membrane formation remains the most frequent cause of failure of vitreoretinal surgery, even after intravitreal daunomycin.

This study shows that intravitreal daunomycin resulted in the prolonged expression of P-glycoprotein in those cells that form recurrent epiretinal membranes in PVR. The intensity of staining was strongest within 8 months after daunomycin treatment and faded thereafter. Other factors like age, gender, type of previous surgery, or cellularity of the specimens did not correlate with the intensity or pattern of P-glycoprotein expression. The dominant cell type expressing the mdr-1 gene in PVR membranes was cytokeratin-positive and thus tentatively identified as RPE origin. Colocalization studies also revealed expression of P-glycoprotein in glial cells and macrophages in a few cases. This is consistent with previous studies that reported P-glycoprotein expression by glia-derived tumor cells and macrophages. Occasionally, the cellular origin of P-glycoprotein-positive cells in the PVR membranes could not be identified; they were negative for either RPE, glia, or monocyte-macrophage markers. These cells are probably fibroblasts that have been detected in PVR membranes and assume an MDR phenotype under certain conditions. The faint P-glycoprotein expression in two patients not previously treated with PVR was localized to RPE cells, suggesting that multiple stimuli may induce mdr-1 gene expression by RPE cells in vivo.

The major role of RPE cells in P-glycoprotein expression in PVR membranes prompted us to investigate a possible chemotherapy-dependent induction of P-glycoprotein expression in human RPE cells in vitro.

Untreated RPE cells before the first passage or passaged twice in vitro expressed mdr-1 mRNA but no P-glycoprotein (Fig. 3). Exposure to daunomycin for 2 weeks enhanced mdr-1 mRNA expression moderately but, more importantly, induced strong expression of P-glycoprotein. Thus, expression of the MDR phenotype in human RPE cells appeared to be regulated by the rate of mRNA translation rather than transcription. It is largely unknown whether this is also the mechanism in other cell lines used to assume the MDR phenotype. Induction of P-glycoprotein expression by daunomycin was associated with enhanced resistance to the drug. This was probably mediated by P-glycoprotein, because resistance was overcome by a classical MDR inhibitor, verapamil, as previously reported for other cell types. An EC50 shift for drug toxicity mediated by P-glycoprotein may be difficult to overcome in vivo, because significantly higher drug doses are required to achieve the same degree of growth inhibition. Corresponding increases in daunomycin doses may result in unacceptable daunomycin-related retinal toxicity. Instead of increasing intravitreal doses of daunomycin, the local or systemic application of a MDR inhibitor such as verapamil together with daunomycin or a MDR-independent antiproliferative agent other than daunomycin might be considered for the treatment of recurrent PVR after intravitreal daunomycin treatment.

Although verapamil reliably reverses the MDR phenotype in vitro, its systemic clinical application is unpredictable because of toxicity to normal cells and to the cardiovascular system at plasma concentrations of 1 to 6 µM, the concentrations required to block P-glycoprotein activity. This might be overcome by the combination of several MDR blockers at subtoxic concentrations. Of note, alternative antimitotic drugs for PVR recurrence after intravitreal daunomycin like taxol, colchicine, and Adriamycin are also subject to MDR-type drug resistance. In contrast, the effects of 5-fluorouracil and BCNU (carmustine) are probably not affected by P-glycoprotein and may therefore be antiproliferative agents of choice.

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References


