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Involvement of P-glycoprotein in the release of cytokines from peripheral blood mononuclear cells treated with methotrexate and dexamethasone

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Abstract

P-glycoprotein (P-gp), a product of the MDR1 gene, is an important factor in the turnover of many drugs and xenobiotics. Recent reports have suggested that P-gp can also be involved in the transport of cytokines. The aim of this study was to examine the role of P-gp in cytokine release from phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (MNCs) as well as in the release of cytokines from MNCs treated with methotrexate (MTX) and dexamethasone (DEX). The study was carried out on PHA-stimulated MNC from 10 healthy subjects. Flow cytometry was applied to measure interleukin (IL)-2, IL-4, IL-6, IL-10, interferon (IFN)-γ and tumour necrosis factor (TNF)-α levels in the culture supernatants. In the experiments verapamil (VER) and P-gp specific monoclonal antibodies (mAb) (clone 17F9) were used to inhibit P-gp function. P-gp inhibitors suppressed the release of IL-2, IL-4, IFN-γ and TNF-α from PHA-stimulated MNC, whereas release of IL-6 and IL-10 remained unaffected. VER and mAb significantly decreased the release of IL-2, IL-4, TNF-α and INF-γ in MNC cultures treated with MTX or DEX. The results of this study suggest that P-gp may be involved in the transmembrane transport of some cytokines. Moreover, it seems that blocking of P-gp function may influence the release of some cytokines from MNCs, displaying an additive inhibitory effect to DEX and MTX.

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Introduction

Multidrug resistance (MDR) is defined when tumour cells develop resistance to a number of structurally and functionally unrelated chemotherapeutic agents. This phenomenon is mediated via the MDR gene, which encodes P-glycoprotein (P-gp), a large transport membrane protein responsible for the decreased accumulation of cytotoxic agents in resistant tumour cells (Fojo et al 1987). Tissue distribution suggests a physiological role for P-gp in the secretion of metabolites and natural xenobiotics (Klimecki et al 1994; Lum & Gosland 1995). Modulation of MDR1 expression in normal cells may influence the activity and bioavailability of drugs and xenobiotics (Varma et al 2003).

Immunosuppressive drugs are often used in the treatment of autoimmune disorders to suppress autoreactive T cells. Mechanisms by which different immunosuppressive drugs interfere with T cell activation and proliferation are diverse. Glucocorticosteroids reduce cytokine production at three levels – interference with transcription factors binding to DNA, destabilization and degradation of mRNA and inhibition of cytokine synthesis through decreased elongation of proteins of the ribosome (Auphan et al 1997). Methotrexate is a folate antagonist used in the therapy of malignant disorders and low doses of methotrexate were introduced for the treatment of rheumatoid arthritis because of its presumed antiproliferative properties (Genestier et al 1998). The pharmacological effects of methotrexate are based on several mechanisms leading to the modulation of cell activation, secretion of cytokines and induction of apoptosis of T cells.

Due to postulated P-gp involvement in the transmembrane transport of endogenous proteins, it is also suggested that it has a potential role in the transport of cytokines (Drach

et al 1996; Raghu et al 1996). Nevertheless the role of P-gp in the release of cytokines from cells treated with immunosuppressive drugs has to be elucidated. It is possible that P-gp might modulate the anti-inflammatory effects of methotrexate and glucocorticosteroids, influencing the cellular concentration of these drugs, especially in lymphocytes releasing the majority of cytokines, although it does not seem that methotrexate or glucocorticosteroids modulate the release of cytokines influencing P-gp activity. Nevertheless, an interaction between P-gp and methotrexate and glucocorticosteroids, as well as other transporters, might influence its effects.

Previous studies showed that the anti-inflammatory properties of methotrexate and glucocorticosteroids are mediated by their influence on interleukin (IL)-2, IL-4, IL-6, IL-10, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ release. Therefore, the objective of this study was to examine the involvement of P-gp and its inhibition in the release of these cytokines from phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (MNCs), as well as peripheral blood MNCs treated with methotrexate and dexamethasone.

Materials and Methods

Cell preparation

Mononuclear cells were isolated from the peripheral blood of 10 healthy subjects using Ficoll Paque. Their viability was assayed by a trypan blue exclusion test. The cells (2×10^6) were stimulated with PHA (concentration 5 $\mu g\,mL^{-1}$) and incubated for 24 h at 37°C, 5% CO_2, in RPMI 1640 medium.

Verapamil (Isoptin; Knoll, D) and P-gp specific monoclonal antibodies (mAb), clone 17F9 (Beckton Dickinson, San Jose, USA) were used in the experiments to inhibit P-gp function. The cells were incubated with increasing concentrations of verapamil, mAb, methotrexate (Rhone-Poulenc F) or dexamethasone (Dexaven; Jelfa, PL) for 24 h. Levels of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α in the culture supernatants were quantified by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA) using the Th1/Th2 kit (Beckton Dickinson, San Jose, CA) according to instructions of the manufacturer.

The study was approved by the local ethics committee and written informed consent was obtained from all subjects.

Statistical analysis

Data were expressed as mean values ± s.d. The distributions of cytokine concentrations were different from normal distribution (Shapiro-Wilk's test), therefore repeated-measures Friedman analysis of variance followed by Wilcoxon's test were applied.

Results

Release of cytokines from PHA-stimulated MNCs in the presence of P-gp inhibitors

The cytokine concentrations after 24 h of stimulation with PHA are shown in Table 1. The treatment of MNCs with both verapamil (1–50 μM) and mAb (1–10 $\mu g\,mL^{-1}$) resulted in a significant reduction in IL-2, IL-4, IFN-γ and TNF-α concentrations in the culture supernatants. There were no statistically significant differences in the release of IL-6 and IL-10.

Effect of methotrexate and dexamethasone on the release of cytokines by PHA-stimulated MNCs

To establish the effect of methotrexate and dexamethasone on release of cytokines from PHA-stimulated MNCs, peripheral blood MNCs were stimulated with PHA

Table 1 The effect of verapamil (VER), anti-Pgp monoclonal antibodies (mAb), methotrexate (MTX) and dexamethasone (DEX) on the release of cytokines from PHA-stimulated peripheral blood mononuclear cells (MNCs)

Treatment		IL-2 ($pg\,mL^{-1}$)	IL-4 ($pg\,mL^{-1}$)	IL-6 ($pg\,mL^{-1}$)	IL-10 ($pg\,mL^{-1}$)	IFN-γ ($pg\,mL^{-1}$)	TNF-α ($pg\,mL^{-1}$)
Control		32 ± 18	42 ± 21	4125 ± 1872	105 ± 59	184 ± 67	239 ± 108
VER (μM)	1	30 ± 16	41 ± 19	4020 ± 1715	106 ± 57	182 ± 71	235 ± 98
	5	24 ± 11*	35 ± 15*	3950 ± 1923	104 ± 54	154 ± 65*	181 ± 74*
	10	20 ± 8**	27 ± 11**	4080 ± 1862	108 ± 57	139 ± 65**	175 ± 72**
	50	18 ± 8**	25 ± 11**	4035 ± 1890	103 ± 65	110 ± 47**	162 ± 71**
mAb ($\mu g\,mL^{-1}$)	1	28 ± 15*	37 ± 18*	4012 ± 1852	112 ± 64	161 ± 72*	220 ± 95*
	5	20 ± 8**	28 ± 11**	4028 ± 1993	103 ± 59	122 ± 54**	163 ± 75**
	10	16 ± 6**	23 ± 9**	4053 ± 1874	101 ± 62	102 ± 45**	147 ± 67**
MTX (μM)	0.1	28 ± 11*	43 ± 19	3751 ± 1548*	107 ± 61	144 ± 56**	218 ± 98*
	1	22 ± 8**	44 ± 21	3211 ± 1458**	110 ± 54	118 ± 96**	180 ± 83**
	10	17 ± 6**	43 ± 18	3123 ± 1363**	106 ± 55	93 ± 42**	172 ± 71**
DEX (μM)	0.01	25 ± 11*	31 ± 13*	3514 ± 1547*	108 ± 54	118 ± 53*	180 ± 75*
	0.1	18 ± 6*	19 ± 7*	2798 ± 1452*	106 ± 48	72 ± 31*	142 ± 67*
	1	7 ± 2*	12 ± 4*	2415 ± 1261*	109 ± 58	50 ± 18*	92 ± 34*

Values are means ± s.d. *$P < 0.05$, **$P < 0.001$ vs control (Friedman analysis of variance followed by Wilcoxon's test).

($5\,\mu g\,mL^{-1}$) in the presence of methotrexate (0.1–10 μM) or dexamethasone (0.01–1 μM) for 24 h.

As shown in Table 1, methotrexate significantly decreased the release of IL-2, IL-6, IFN-γ and TNF-α, whereas no significant effect on the release of IL-4 and IL-10 was observed.

Treatment with dexamethasone resulted in decreased concentrations of IL-2, IL-4, IL-6, IFN-γ, and TNF-α.

Effect of P-gp inhibition on the release of cytokines from PHA-stimulated MNCs in the presence of methotrexate and dexamethasone

To study the effect of P-gp inhibition on the release of cytokines from PHA-stimulated MNCs treated with methotrexate and dexamethasone, peripheral blood MNCs were stimulated with PHA ($5\,\mu g\,mL^{-1}$) in the presence of methotrexate (1 μM) or dexamethasone (0.1 μM) and P-gp inhibitors — verapamil (1–50 μM) or mAb (1–$10\,\mu g\,mL^{-1}$). The level of cytokines was measured in the supernatants after 24 h.

As shown in Table 2, verapamil and mAb significantly decreased the release of IL-2, IL-4, IFN-γ and TNF-α in MNC cultures treated with methotrexate and dexamethasone. The release of IL-6 and IL-10 remained unaffected.

Discussion

In this study we investigated the hypothesis that P-gp is involved in the transmembrane transport of some cytokines and whether P-gp inhibition by verapamil and anti-P-gp specific antibodies may influence the release of cytokines from cells treated with methotrexate and dexamethasone. Verapamil has been shown to inhibit many members of the ABC family of transporters and other unrelated proteins. Because the data from verapamil studies supports a hypothesis for the involvement of ABC transporters, but not specifically P-gp, in these experiments the clone 17F9 of anti-P-gp specific antibodies was used to inhibit specifically P-gp function. This clone was used in our previous studies and by other investigators (Shi et al 1995; Pawlik et al 2005).

The inhibition of P-gp expression both by verapamil and P-gp specific monoclonal antibodies resulted in significantly decreased release of IL-2, IL-4, IFN-γ and TNF-α from PHA-stimulated, as well as methotrexate- and dexamethasone-treated, mononuclear cells. The release of IL-6 and IL-10 remained unaffected.

The reports evaluating the involvement of P-gp in transmembrane transport of cytokines are controversial. Raghu et al (1996) showed that treatment of PHA-activated peripheral blood lymphocytes with P-gp specific monoclonal antibodies resulted in a significant reduction of IL-2 levels in cultures. Drach et al (1996) studied the involvement of P-gp in the transmembrane transport of IL-2, IL-4, IL-6 and IFN-γ in PHA-stimulated T lymphocytes from healthy subjects. It was revealed that the release of IL-2, IL-4 and IFN-γ was significantly inhibited by P-gp inhibitors (i.e. verapamil, tamoxifen and P-gp-specific monoclonal antibodies), whereas release of IL-6 remained unaffected. The authors concluded that P-gp participates in the transport of IL-2, IL-4 and IFN-γ in peripheral blood lymphocytes.

Gollapudi et al (2000) examined the secretion of IL-2 by anti-CD3-stimulated P-gp(+) and P-gp(−) subsets of CD4+ and CD8+ T cells from healthy subjects as well as the release of IL-2, IL-4, IL-10 and IFN-γ in mice. The authors suggested that P-gp was not required for secretion

Table 2 The effect of verapamil (VER) and anti-Pgp monoclonal antibodies (mAb) on the release of cytokines from PHA-stimulated peripheral blood mononuclear cells (MNCs) treated with 1 μM methotrexate (MTX) or 0.1 μM dexamethasone (DEX)

Treatment		IL-2 ($pg\,mL^{-1}$)	IL-4 ($pg\,mL^{-1}$)	IL-6 ($pg\,mL^{-1}$)	IL-10 ($pg\,mL^{-1}$)	IFN-γ ($pg\,mL^{-1}$)	TNF-α ($pg\,mL^{-1}$)
Control 1 μM MTX		22 ± 8	44 ± 21	3211 ± 1458	110 ± 54	118 ± 96	180 ± 83
VER (μM) + 1 μM MTX	1	21 ± 9	43 ± 20	3154 ± 1512	108 ± 59	115 ± 51	177 ± 92
	5	18 ± 7*	37 ± 15*	3242 ± 1484	105 ± 47	98 ± 42*	156 ± 71*
	10	15 ± 6**	28 ± 11**	3121 ± 1489	114 ± 61	92 ± 43**	148 ± 67*
	50	13 ± 5**	26 ± 11**	3156 ± 1483	112 ± 54	84 ± 32**	139 ± 61**
mAb ($\mu g\,mL^{-1}$) + 1 μM MTX	1	19 ± 8*	40 ± 17*	3273 ± 1482	107 ± 58	97 ± 34*	164 ± 67*
	5	15 ± 7**	30 ± 12**	3140 ± 1517	112 ± 48	89 ± 32*	148 ± 58*
	10	12 ± 4**	23 ± 9**	3133 ± 1459	104 ± 48	85 ± 37**	132 ± 55**
Control 0.1 μM DEX		18 ± 6	19 ± 7	2798 ± 1452	106 ± 48	72 ± 31	142 ± 67
VER (μM) + 0.1 μM DEX	1	17 ± 7	18 ± 7	2853 ± 1418	112 ± 52	70 ± 28	138 ± 97
	5	16 ± 6*	15 ± 6*	2897 ± 1299	108 ± 48	62 ± 23*	123 ± 42*
	10	14 ± 4**	14 ± 5**	2872 ± 1281	105 ± 52	57 ± 21**	105 ± 38**
	50	10 ± 3**	13 ± 3**	2750 ± 1246	110 ± 50	53 ± 19**	89 ± 28**
mAb ($\mu g\,mL^{-1}$) + 0.1 μM DEX	1	14 ± 5*	15 ± 7*	2754 ± 1252	108 ± 52	65 ± 21*	121 ± 47*
	5	12 ± 3**	12 ± 4**	2814 ± 1210	102 ± 46	54 ± 18**	106 ± 42**
	10	9 ± 3**	10 ± 3**	2823 ± 1286	100 ± 45	48 ± 17**	84 ± 35**

Values are means ± s.d. *$P < 0.05$, **$P < 0.001$ vs control (Friedman analysis of variance followed by Wilcoxon's test).

of IL-2, IL-4, IL-10 and IFN-γ in mice and IL-2 secretion in man.

Previous studies showed that the anti-inflammatory properties of methotrexate and glucocorticosteroids were associated with modulation of cytokine release by immunocompetent cells (Auphan et al 1997; Constantin et al 1998; Neurath et al 1999). Our results in this study indicate that inhibition of P-gp activity modulates the anti-inflammatory properties of methotrexate and dexamethasone associated with decreased release of some cytokines from PHA-stimulated MNCs.

Dexamethasone is known to be a substrate for P-gp. The excretion of both endogenous and exogenous steroids is mediated by P-gp (Cordon-Cardo et al 1990). In the mdr-gene knockout mouse model, the absence of P-gp is associated with higher concentrations of dexamethasone in the brain (Schinkel et al 1995). Moreover, it has been shown that the mdr1b P-glycoprotein in a mouse thymoma cell line can confer resistance against this drug (Bourgeois et al 1993). Verapamil was found to enhance the absorption of methylprednisolone in the jejunum of rats (Saitoh et al 1998). Accumulation of hydrocortisone is reduced in mdr-pituitary tumour cells and is restored after blockade with P-gp specific monoclonal antibodies (Nelson & Hinkle 1992).

Although methotrexate is not known to be a substrate for P-gp, several studies demonstrated the involvement of P-gp in methotrexate resistance. Norris et al (1996) examined the expression of P-gp in a series of leukaemia sublines resistant to methotrexate. The authors demonstrated increased expression of MDR1 messenger RNA and increased P-gp expression in those sublines. Resistance to methotrexate was reversed by P-gp specific monoclonal antibodies as well as by verapamil or ciclosporin. In a second study, de Graf et al (1996) hypothesized that P-gp may mediate methotrexate resistance in cells with deficient carrier-mediated methotrexate uptake. The authors reported that insertion of recombinant retrovirus expressing the human MDR1 gene resulted in increased survival of resistant cells. These studies demonstrate the potential of P-gp over-expression in conferring resistance to methotrexate.

In our previous study we demonstrated the involvement of genetically determined P-gp over-expression in the resistance to methotrexate and glucocorticosteroids in patients with rheumatoid arthritis (Pawlik et al 2004). The probability of remission of rheumatoid arthritis symptoms after therapy with these drugs was significantly higher in patients with MDR1 3435 TT genotype (low P-gp expression) than in subjects with MDR1 3435 CC and CT genotypes (high P-gp expression).

The results of this study suggest that P-gp may be involved in the transmembrane transport of some cytokines, also in the presence of immunosuppressive drugs such as methotrexate and dexamethasone. It seems that blocking P-gp function may influence the release of some cytokines from MNCs, having an additive inhibitory effect to dexamethasone and methotrexate. Nevertheless, the elucidation of the precise mechanisms of this interaction requires further studies.

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