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Biochemical Pharmacology 65 (2003) 1419–1425
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Multidrug resistance correlates with overexpression of Muc4 but inversely with P-glycoprotein and multidrug resistance related protein in transfected human melanoma cells

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Received 10 July 2002; accepted 25 September 2002

Abstract

Due to the size, glycosylation, and location in the plasma membrane of the sialomucin complex Muc4, which has been implicated in ErbB2 signaling, in the repression of apoptosis and cell adhesion, and in tumor metastasis, studies were initiated to determine whether its presence could influence cell sensitivity to anticancer drugs. Growth inhibition assays using melanoma cell lines that either express the glycoprotein ($Muc4^+$) or do not ($Muc4^-$) showed that Muc4 renders cells resistant to taxol, doxorubicin, vinblastine, rhodamine 123, and 2-deoxyglucose. When treated with various concentrations of doxorubicin, $Muc4^+$ cells were blocked less frequently in G_2 and underwent less DNA fragmentation (apoptosis and/or necrosis) than $Muc4^-$ cells. All of the drugs tested (except for 2-deoxyglucose) are well recognized by P-glycoprotein-mediated multidrug resistance 1 (MDR1) and to a lesser degree by multidrug resistance related protein 1 (MRP1) transporters. Therefore, transporter gene expression in these cells was assayed. Surprisingly, $Muc4^+$ cells expressed lower levels of both transporter genes than $Muc4^-$ cells. Moreover, rhodamine 123 was retained more highly in the $Muc4^+$ than in the $Muc4^-$ cells, demonstrating that these transporters are functional. Overall, these results indicate that although $Muc4^+$ cells express less MDR1 and MRP1, they are more resistant to drugs recognized by these transporters.

Keywords: Muc4; Multidrug resistance; P-gp; MRP; Melanoma; Rhodamine 123

1. Introduction

Muc4 (also known as the sialomucin complex) is a heterodimeric membrane glycoprotein originally isolated from highly metastatic ascites rat mammary adenocarcinoma cells [1]. It is composed of a mucin subunit, ASGP-1 [2], attached to the membrane via a transmembrane subunit, ASGP-2 [3]. The complex is derived from a single gene [4,5] of the mucin family [6,7] and synthesized as a high molecular weight precursor, which is cleaved during transit to the cell surface [8]. Two different functions have been ascribed to Muc4. First, the large rigid mucin subunit ASGP-1 acts as an anti-adhesion [9] or anti-recognition [10] factor to block access of other cells, particles, and large molecules [10] to the cell surface. This function appears to play a role in the protection of certain accessible and vulnerable normal epithelia that express it [11], but also can prevent immune cell killing of tumor cells in which Muc4 is overexpressed [10]. Second, the transmembrane subunit ASGP-2 acts as a novel intramembrane ligand for the receptor tyrosine kinase ErbB2, inducing specific ErbB2 phosphorylation and potentiating the neuregulin-stimulated phosphorylation of both ErbB2 and ErbB3 [12].

Muc4 is large, extensively glycoslyated, and located in the plasma membrane [1]. Since the glycoproteins, most notably P-gp and MRP, responsible for multidrug resistance in a wide variety of tumor and normal cells [13] are similarly located, studies were initiated to determine whether the presence of this complex could influence cell

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Abbreviations: Rho 123, rhodamine 123; DOX, doxorubicin; Muc4, sialomucin complex; ASGP, ascites sialoglycoprotein; P-gp, P-glycoprotein; MDR, multidrug resistance; MRP, multidrug resistance related protein; RT–PCR, reverse transcriptase–polymerase chain reaction.

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doi:10.1016/S0006-2952(03)00086-8

sensitivity to anti-cancer drugs. To accomplish this, a human melanoma cell line (A375) was transfected with the *Muc4* gene that was expressed under the control of a tetracycline-responsive inducible promoter [9]. The cells undergo a distinct morphological change and lose adhesion to their substratum and to neighboring cells in culture when Muc4 expression is induced at a high level (100-fold over control) within 48 hr after tetracycline is removed from the medium [9]. Thus, the tetracycline-regulated Muc4 expression system provides a convenient cellular model to determine the importance of this glycoprotein to antitumor drug toxicity. Using this system, growth inhibition activities of the chemotherapeutic agents taxol, DOX, and vinblastine, as well as the mitochondrial-localizing dye Rho 123, and the glycolytic inhibitor 2-deoxyglucose, were investigated. These compounds (except for 2-deoxyglucose) were selected because they are all recognized by the MDR glycoproteins, P-gp and MRP, which actively transport these drugs out of tumor and normal cells [13] and consequently render them resistant.

2. Materials and methods

2.1. Cell culture

The human melanoma cell line A375 transfected with the *Muc4* gene, as previously described [9], was grown in complete medium containing 10% fetal bovine serum (FBS), 100 units/mL of penicillin–streptomycin, 0.4 mg/mL of Geneticin (G418), and 0.17 mg/mL of Hygromycin B with or without 2.4 μg/mL of tetracycline. To induce Muc4 expression, the cells ($Muc4^+$) were grown in medium without tetracycline for 48 hr [9]. To inhibit expression of Muc4, cells were maintained in medium with tetracycline and showed significantly reduced levels of the glycoprotein ($Muc4^-$).

2.2. Analysis of Muc4 expression on cell surface

Cells were collected and resuspended in PBS (Dulbecco's phosphate-buffered saline without calcium) containing 3% BSA. Cell suspensions were incubated with anti-ASGP-2 mAb 4F12 [14], followed by incubation with fluorescein isothiocyanate-conjugated secondary antibodies (Sigma). The fluorescence intensity was determined by FACScan analysis.

2.3. Growth inhibition assay

Cells, 24 hr after seeding, were treated with drugs (either Rho 123, taxol, DOX, vinblastine, or 2-deoxyglucose; purchased from the Sigma Chemical Co.) for 72 hr, trypsinized, and counted using the trypan blue exclusion method. The concentration that inhibited cell growth by 50% was calculated (IC_{50}) for each drug.

2.4. Cell cycle analysis

Cells were treated with drugs for 72 hr, harvested at 2×10^5, and fixed in 1 mL of ice-cold 70% ethanol overnight at 4°. The mixture was centrifuged at 1500 *g* for 5 min at room temperature, and the supernate was removed. The pelleted cells were stained with 50 μg/mL of propidium iodide and 100 units/mL of RNase. The fraction of subdiploid cells with oligonucleosomal DNA degradation, characteristic of cell death, apoptosis and/or necrosis, was quantified by flow cytometric analysis.

2.5. Analysis of MDR1 and MRP1 gene expression by PCR

Total RNA was purified by acid-guanidinium isothiocyanate/phenol chloroform extraction. One microgram of RNA was used for cDNA synthesis as follows. RNA was heated (70° for 5 min), chilled on ice, and then incubated with 200 units of M-MLVH reverse transcriptase, 500 nM dNTP, 10 pM random primer, and RT buffer (Gibco-BRL) for 1 hr at 37°. For MDR1, the primers were the *mdr1* specific sequences GGAGTGTCCGTGGATCACAAG (residues 1909–1930) and TGTTCAGGATCATCAATTCTTGT (residues 2218–2241). These primers were selected at regions that are only 36.4 and 37.5% similar to the corresponding region of *mdr-3* cDNA. Thus, they should not recognize the *mdr-3* gene. The resulting PCR product from these primers was 232 bp. For MRP1, the primers were the *mrp1* specific sequence TGGTCATCAGCAGCATCGTG (sense primer) and GCCTGTATCACGGACCTGTAA (anti-sense primer). The resulting PCR product from this primer was 420 bp. GADPH was used as the internal standard, and the amplimers used for GADPH were CCACCACCCTGTTGCTAGCC (antisense) and GTCTTGACCACCCAGGAGAAGGC (sense). These primers yield a 676 bp PCR product. The PCR was performed using the cDNA in PCR buffer according to the Perkin-Elmer Cetus protocol. Added to this mixture were 0.2 mM concentrations each of dATP, dCTP, dGTP, and dTTP, 30 nM concentrations of the 5′ and 3′ primers, and 1.5 units of *Taq* polymerase. Amplification was performed in sequential cycles at 94° for 30 sec, 55° for 1 min, and 72° for 2 min for 30 cycles using a Perkin-Elmer thermocycler. The PCR product was electrophoresed in 1× TAE (0.04 M Tris–acetate, 0.001 M EDTA) buffer on a 2% Nusieve agarose/1% agarose gel. The gel was stained with ethidium bromide and photographed.

2.6. P-gp function assay

Cells were treated with 0.5 μg/mL of Rho 123 with or without verapamil at 10 μg/mL for 10 min, then rinsed three times with Hanks' Balanced Salt Solution with or without verapamil at 10 μg/mL, and kept in fresh medium with or without verapamil for 4 hr at 37°. Rho

123 accumulation/retention in cells was detected by flow cytometry.

2.7. Caspase-9 assay

A caspase-9/Mch6 Fluorometric Assay Kit and the apoptosis inducer actinomycin D were purchased from Biovision, Inc. Apoptosis was induced in cells by treating with 10 μM actinomycin D for 24 hr. A culture without induction was incubated as a control. Cells were counted, and 2×10^6 were pelleted. Cells were resuspended in 50 μL of cell lysis buffer and incubated on ice for 10 min. Following this incubation, cells were centrifuged for 1 min at 4° in a microcentrifuge (10,000 *g*). Supernatant (cytosolic extract) was transferred to a fresh tube and put on ice. Protein concentration was assayed, and 100–200 μg protein was diluted in 50 μL of cell lysis buffer for each assay. Fifty microliters of reaction buffer (containing 10 mM dithiothreitol) and 5 μL of the 4 mM LEHD-NA substrate (200 μM final concentration) were added and incubated at 37° for 1 hr. Samples were read in a microtiter plate reader. Background readings from cell lysates and buffers were subtracted from the readings of both induced and uninduced samples before calculating caspase-9 activity.

3. Results

3.1. Muc4 expression levels in transfected cells with and without tetracycline

Induction of Muc4 and analysis of the expression levels on the cell surface in the transfectants were monitored by FACScan analysis. Forty-eight hours after tetracycline was removed, Muc4 was found to be strongly expressed on the cell surface of A375 transfectants in the absence of tetracycline compared to the A375 cells grown in tetracycline medium, as previously reported [9].

3.2. Growth inhibition of Muc4$^+$ and Muc4$^-$ cells with various MDR drugs

Growth inhibition studies demonstrated that the IC_{50} values in A375 Muc4$^+$ cells were 6.0-, 5.0-, 2.0-, 1.7-, and > 3.2-fold greater than in Muc4$^-$ cells, when treated with taxol, Rho 123, DOX, vinblastine, or 2-deoxyglucose, respectively (Table 1). Thus, cell resistance to all of the drugs tested correlates with the expression of Muc4.

Table 1
Growth inhibition of Muc4$^+$ and Muc4$^-$ cells by various drugs (IC_{50})

Drugs	IC_{50} (μg/mL)		
	Muc4$^-$	Muc4$^+$	Resistance ratio
Taxol	0.011 ± 0.002	0.06 ± 0.013	6
Rho 123	2.66 ± 0.31	13.2 ± 2.2	5
Doxorubicin	0.0024 ± 0.0005	0.0048 ± 0.001	2
Vinblastine	0.0036 ± 0.0021	0.006 ± 0.0024	1.7
2-Deoxyglucose	620	> 2000	> 3.2

Values are means ± SD of triplicate samples (representative of > 3 experiments per drug).

3.3. MDR1 (P-gp) and MRP1 mRNA levels in Muc4$^+$ and Muc4$^-$ cells

Since all of the compounds used in this study, except 2-deoxyglucose, have been shown previously to be recognized by the major drug transporters responsible for multidrug resistance, P-gp and MRP, the expression of each of these genes was assayed by RT–PCR. Both *MDR1* (P-gp) and *MRP1* gene expression in Muc4$^+$ cells was found to be lower than in Muc4$^-$ cells (Fig. 1). This result is surprising because the expression of the drug-transporting pumps in these two cell variants does not correlate with drug resistance, i.e. Muc4$^-$ cells express more MDR1 and MRP1 than Muc4$^+$ cells, yet are more sensitive to the MDR-recognizable drugs.

3.4. Correlation of increased levels of P-gp and MRP in Muc4$^-$ cells with decreased retention of Rho 123

To determine whether the higher expression of the *MDR1* and *MRP1* genes in Muc4$^-$ cells correlates with lower drug accumulation, the intracellular amounts of Rho 123 were assayed. Figure 2 shows that approximately 30% of Muc4$^-$ cells treated with Rho 123 for 10 min at

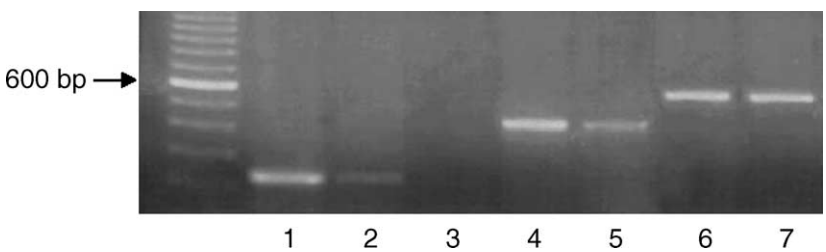

Fig. 1. Analysis of *MDR1* and *MRP1* gene expression in Muc4$^+$ and Muc4$^-$ cells by RT–PCR. The left-hand lane represents DNA size markers. Lanes 1–7 are as follows: 1, MDR1 PCR product (control); 2, MDR1 PCR product in Muc4$^-$ cells; 3, MDR1 PCR product in Muc4$^+$ cells; 4, MRP1 PCR product in Muc4$^-$ cells; 5, MRP1 PCR product in Muc4$^+$ cells; 6 and 7, GADPH PCR product in Muc4$^-$ and Muc4$^+$ cells, respectively (internal RNA controls).

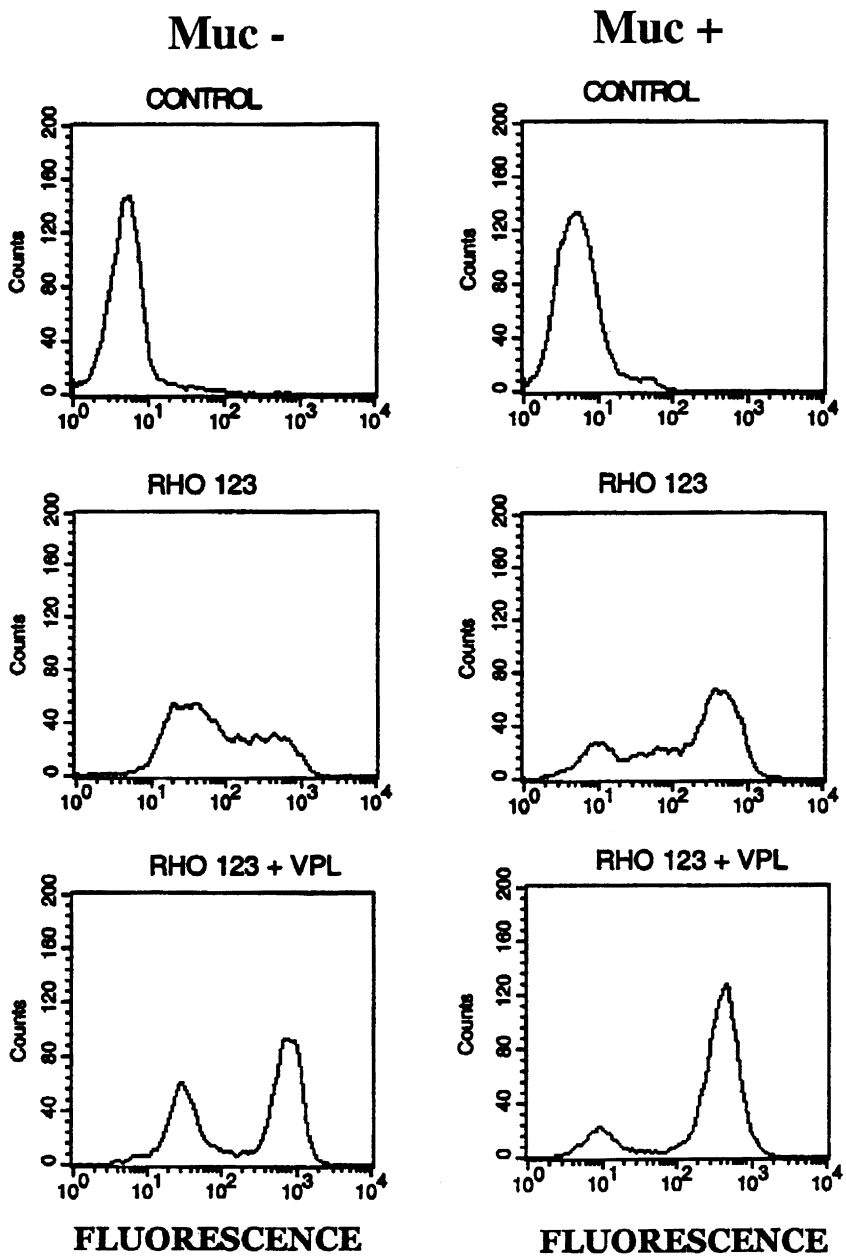

Fig. 2. Rho 123 retention in the presence or absence of verapamil in Muc4$^-$ and Muc4$^+$ expressing cells. Note that significantly less Muc4$^-$ cells (about 30%) than Muc4$^+$ cells (about 65%) treated with Rho 123 (0.5 μg/mL) for 10 min stained at > 102 fluorescence units. When cells were co-treated with Rho 123 and verapamil (VPL, 10 μg/mL), the number of cells staining with > 102 fluorescence units increased in both Muc4$^-$ and Muc4$^+$ cells. In the upper panels, untreated control cells show auto-fluorescence levels.

0.5 μg/mL had a fluorescence intensity greater than 102. When co-treated with the P-gp and MRP blocker verapamil (10 μg/mL), the number of cells staining at this intensity increased to 65%. In contrast, Muc4$^+$ cells similarly treated with Rho 123 showed a significantly higher number of cells (65%) staining with fluorescence intensity units greater than 102, and when co-treated with verapamil this number increased to 80%. Thus, drug retention correlated with the expression levels of the drug transporters (Fig. 1), but not with growth inhibition (Table 1).

3.5. Increased cell cycle blockage and DNA fragmentation in Muc4$^-$ versus Muc4$^+$cells when treated with DOX

Since drug accumulation did not correlate with growth inhibition in Muc4$^-$ and Muc4$^+$ cells, the differential effects on cell cycle blockage and apoptosis/necrosis (DNA fragmentation) were analyzed in these cells after treatment with DOX. DNA content analysis of propidium iodide-labeled cells demonstrated that DOX induced a concentration-dependent G_2 block in both cell lines

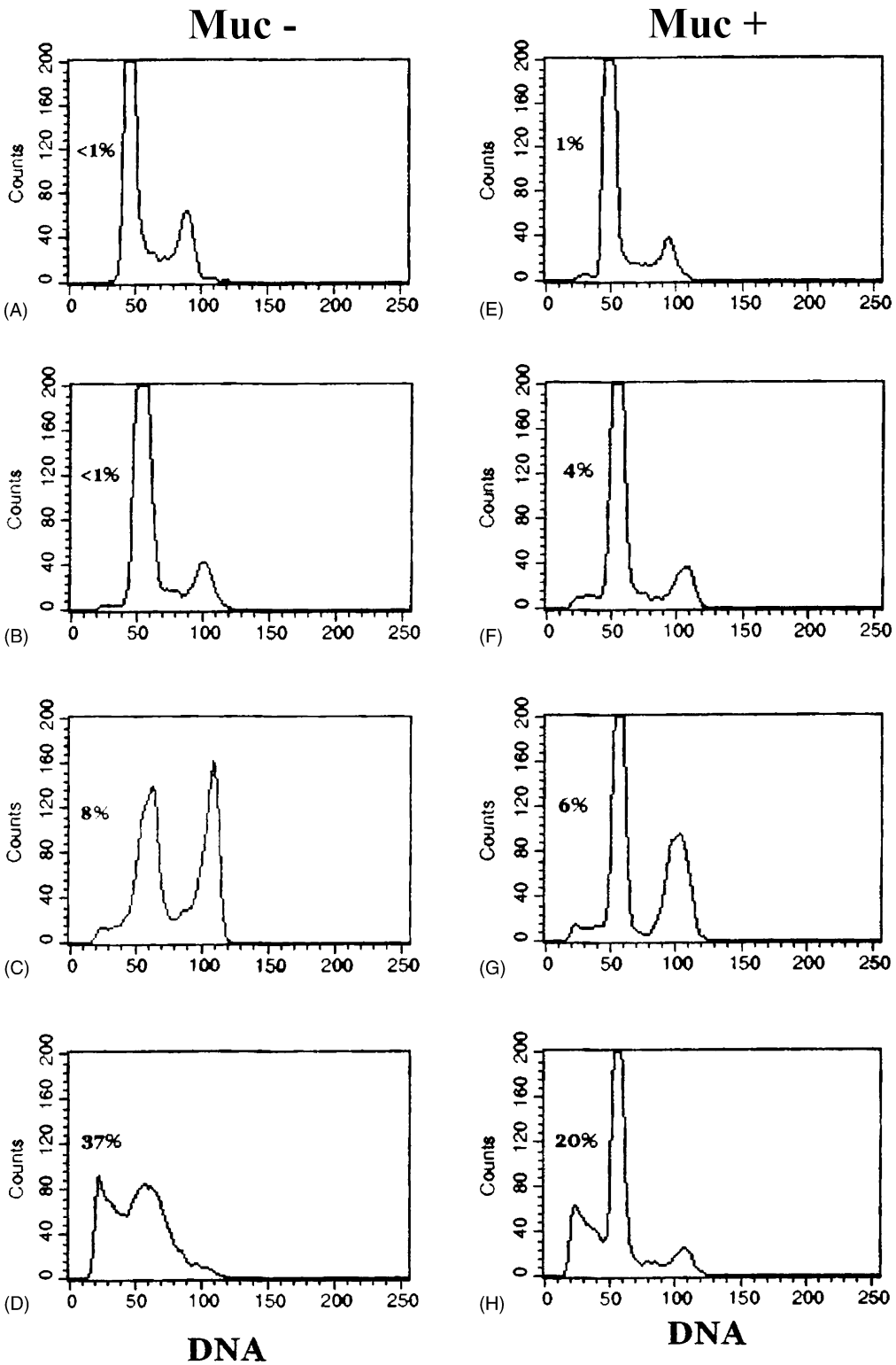

Fig. 3. Determination of cell cycle distribution and apoptosis by DNA content analysis of propidium iodide-labeled Muc4$^-$ and Muc4$^+$ cells treated with different concentrations of DOX for 72 hr. Untreated, A and E; 0.0025 μg/mL, B and F; 0.025 μg/mL, C and G; and 0.125 μg/mL, D and H.

(Fig. 3, A–C, and E–G). Note that slightly more Muc4$^-$ cells than Muc4$^+$ cells were blocked in G_2 (Fig. 3, C and G). At higher concentrations of DOX, 37% of the Muc4$^-$ cells shifted from G_2 to subdiploid (indicative of cell death due to apoptosis and/or necrosis) compared to only 20% of the Muc4$^+$ cells (Fig. 3, D and H). The data indicate that fewer Muc4$^+$ than Muc4$^-$ cells died when treated with DOX.

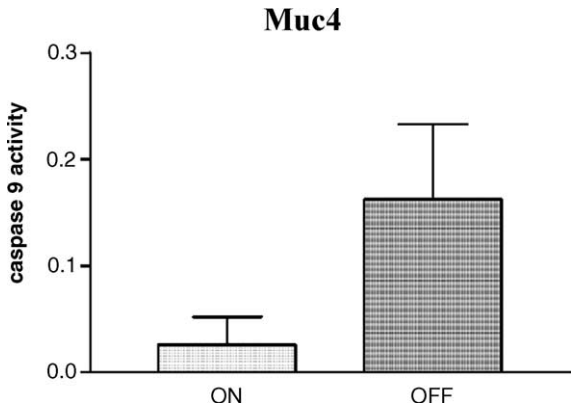

Fig. 4. Inhibition of activation of caspase-9 in Muc4$^+$, but not Muc4$^-$, cells induced to undergo apoptosis by treatment with 10 μM actinomycin D for 24 hr. Values of caspase-9 activity in control cells not challenged with actinomycin D were generally below 0.025. Values are means ± SD (N = 3).

3.6. Muc4 inhibition of caspase-9 activation

The repression of cell death, and the reduced apoptosis in cells expressing Muc4 when they were serum starved as previously noted [15], raise the question of mechanism. To begin to address this question, we analyzed caspases associated with the two primary pathways linked to apoptosis. Caspase-9 and caspase-8 are key elements of the intrinsic and extrinsic pathways, respectively [16]. As shown in Fig. 4, caspase-9 activation was strongly inhibited by expression of Muc4 when cells were treated with the apoptotic inducing agent, actinomycin D. In contrast, Muc4 did not repress activation of caspase-8 when the extrinsic pathway was induced (data not shown).

4. Discussion

Most studies with P-gp and MRP have shown that the presence of these glycoproteins in the plasma membrane of both normal and tumor cells correlates with lowered drug accumulation and consequently increased resistance to numerous drugs [13]. On the other hand, there are no data to suggest that the large glycoprotein complex Muc4, which also resides on the plasma membrane, has similar transporter activity. Our growth inhibition results, however, with the "P-gp and MRP recognizable drugs" taxol, vinblastine, DOX, and Rho 123, indicate that Muc4 overexpression in tumor cells correlates with resistance to these drugs (Table 1). Since Muc4$^+$ cells show reduced expression of the MDR effluxing glycoproteins, MDR1 and MRP1, as compared to their Muc4$^-$ cell counterparts, reduced drug accumulation cannot account for this resistance. Clearly the data indicating that a greater amount of Rho 123 accumulated in a higher percentage of Muc4$^+$ than Muc4$^-$ cells (Fig. 2) demonstrate that MRP and P-gp are indeed functioning in these cell types. Moreover, the fact that a greater percentage of Muc4$^-$ than Muc4$^+$ cells accumulated more Rho 123 when the cells were co-treated with the P-gp and MRP blocker verapamil (Fig. 2) further confirms that Pgp and/or MRP are functional. Similar results were observed when the cells were treated with DOX (instead of Rho 123) for 2 hr at 10 μg/mL (data not shown). Therefore, if lowered drug accumulation due to P-gp and or MRP does not correlate with cellular resistance in these cell types, another mechanism must account for this phenomenon.

Our data with DOX, in which it was found that Muc4$^+$ cells underwent less G_2 blockade and less death (increased DNA fragmentation) than Muc4$^-$ cells when treated with this drug, point to the possibility that Muc4 expression may be related to resistance via reduced apoptosis and/or necrosis. Although from our studies here we cannot distinguish between the two death processes, the data are consistent with previous studies on Muc4 functions which demonstrated that Muc4 could repress apoptosis triggered by serum starvation in these same melanoma cells [15]. Moreover, it was shown that Muc4 potentiated tumor growth in nude mice through an inhibition of apoptosis [15]. We have now shown that Muc4 expression can repress activation of caspase-9 in cells induced to undergo apoptosis. Thus, the results which demonstrate that Muc4$^+$ cells are resistant to 2-deoxyglucose, a compound not known to be recognized by P-gp or MRP, further support the interpretation that reduced apoptosis is at least partly responsible for MDR in cells expressing Muc4.

Previous studies have shown that ErbB2 expression in tumors can lead to MDR [17]. In the case of taxol, the mechanism was proposed to involve the ErbB2-mediated up-regulation of the cell cycle inhibitor $p21^{Cip1}$, which inhibits activation of cdk1/cyclin B, thus blocking its normal pathway to cellular apoptosis [17]. Muc4 may operate by a similar mechanism. Preliminary studies indicate that Muc4 induces specific phosphorylation of ErbB2 on Tyr^{1248}, recognized by anti-phospho-ErbB2, in A375 melanoma cells [18]. Concomitant with this effect is an up-regulation of the cell cycle inhibitor $p27^{Kip}$ [18] and a repression of apoptosis [15]. The specifics of these pathways remain obscure, but they may represent important mechanisms for allowing tumor cells to escape chemotherapy [17]. Since the drugs used in this study have different modes of action, it is difficult to predict whether reduced apoptosis is a common mechanism responsible for resistance to these agents in Muc4$^+$ cells. Studies to evaluate the amount of apoptosis and necrosis that account for toxicity for each of the drugs used here, as well as other non-MDR recognizable compounds, will be the focus of future investigations. Another question that arises from our results and remains to be addressed is whether the reduced expression of P-gp and MRP, concomitant with overexpression of Muc4, is a universal phenomenon or restricted to the melanoma cell line studied here.

Acknowledgments

FACScan analyses were done at the Flow Cytometry Facility of the Sylvester Cancer Center of the University of Miami under the expert supervision of James Phillips. This work was supported by NCI Grants CA37109 to T.J.L. and CA52498 and CA74072 to K.L.C.

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