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The Effect of Pretreatment with Various Mutagens on Glycoconjugates of Plasma Membrane in HeLa Cells

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ABSTRACT : **The present investigation has been performed to elucidate the effect of pretreatment with low dose of ultraviolet radiation (UV), ethyl methanesulfonate (EMS), and bleomycin (BLM) on cell survival and lectin-binding glycoconjugates of plasma membrane in HeLa cells treated with mutagen. The percentage of survival of cells pretreated with 1 mM EMS following treatment with 10 mM EMS was higher than that of cells treated with 10 mM EMS alone. Wheat germ agglutinin (WGA) staining intensity of cells pretreated with 1 mM EMS and subsequently treated with 10 mM EMS was stronger than that of cells treated with 10 mM EMS alone. But, succinylated wheat germ agglutinin (sWGA) staining intensity of cells pretreated with 1 mM EMS and subsequently treated with 10 mM EMS was similar to that of cells treated with 10 mM EMS alone. These results suggest that the acquired resistance to EMS is related to the glycoconjugates containing sialic acid of plasma membrane involved in multidrug resistance or adaptive response in HeLa cells.**

Key Words : Glycoconjugates, Multidrug Resistance, Adaptive Response, HeLa cells

Introduction

It appears that cells have a vast capacity to protect themselves from toxic substances, and the study of multidrug resistance (MDR) or adaptive response has uncovered many of these capacity (Ford and Hait, 1990). One form of drug resistance, termed MDR, is defined that mammalian and tumor cell lines selected for resistance to a single drug have shown cross resistance between drug compounds without structural and functional similarities due to enhanced outward transport of drugs mediated by a membrane glycoprotein "drug transport pump" (Volm *et al.*, 1989; Nare *et al.*, 1994). In multidrug resistant cells, the most frequently reported change is the overexpression of the 170 kDa plasma membrane glycoprotein (P-gp) originally described by Juliano and Ling (1976). The expression of the P-gp is responsible for the resistance of tumor cells to various hydrophobic cytotoxic drugs (Gottesman *et al.*, 1989; Nare *et al.*, 1994). The mechanism of P-gp in the MDR phenotype is as an ATP-dependent efflux pump, actively extruding cytotoxic drugs from the cell (Chen *et al.*, 1986; Gros *et al.*, 1986). Although it has been suggested that P-gp binds directly to many lipophilic cations, it remains unclear whether its broad substrate specificity is mediated by one or by many sites in P-gp (Nare *et al.*, 1994). The increased expression of the surface P-170 alone is not sufficient to account for all complexities of the multidrug resistance phenotype (Kessel and Basmann, 1970; Biedler and Peterson, 1981). A variety of other membrane-associated changes and alterations in cytoplasmic proteins have been found in different drug-resistant cell lines (Kessel and Basmann, 1970; Biedler and Peterson, 1981; Meyers and Biedler, 1981). Several mechanisms have been reported for this form of MDR, such as altered topoisomerase II, increased glutathione-S-transferase (Kim *et al.*, 1991; Batist *et al.*, 1986), and a new membrane transporter, the multidrug resistance-associated protein that found more recently (Zaman *et al.*, 1993; Cole *et al.*, 1994).

On the other hand, the other form of drug resistance, termed adaptive response, is that inducible DNA repair pathways enable cells to display increased deleterious effects of chemical mutagens and radiation (Lindahl and Sedgwick., 1988). Several factors, including reduced druguptake earlier onset of DNA repair, elevated levels of glutathione-S-transferase, and increased expression of P-gp, appear to contribute to resistance in P388 leukemia cells (Deffie *et al.*, 1992). Furthermore, P-gp overexpression, attributable to murine mdr3, was detected only in the

highest-level vincristine-selected cell line, while, the overexpression of multidrug-resistance associated protein was evident in the low-level vincristine-selected cell lines (Slapak *et al,* 1994). And the increased ricin A binding to glycoprotein of 150 and 300 kDa was observed with the increased levels of resistance to adriamycin (Dickstein *et al.,* 1995). Transcription of *mdr* gene and expression of gammaglutamyl transpeptidase are related with the general cellular adaptive response to the environmental changes rather than with carcinogenic process itself (Kim *et al.,* 1991). Therefore, the purpose of this study is to find out the acquired resistance and the changes of glycoconjugates of plasma membrane to ultraviloet radiation (UV), ethyl methanesulfonate (EMS) and bleomycin (BLM) using biotinylated lectin in HeLa cells.

Materials and Methods

Cell Culture

Human cervical carcinoma, HeLa cells were used throughout this investigations. Monolayer cultures of this cell lines were grown at 37°C in humidified 5% CO_2 incubator using Eagle's minimum essential medium (Grand Island Biological Co., Grand Island., N.Y., U.S.A.) supplemented with 10% fetal calf serum and gentamycin (50 μg/ml).

UV Irradiation

Cells were cultured for more than 48 hours in culture dishes prior to UV-irradiation, the growth medium was then removed from the cultures and the cells were washed twice with phosphate buffered saline (PBS). Cells were then exposed to various doses of 254 nm UV from mercury germicidal lamps at an incident dose rate of 1 J/m²/sec. The dose rate was determined by UVX digital radiometer No. A 030848 (San Gabrial, CA 911778 U.S.A.). The fresh medium was added immediately after the irradiation.

Chemical Treatment

Ethyl methanesulfonate (EMS, Tokyo Kasei Co., Tokyo, Japan) or bleomycin (BLM, Nihon Kayaku, Japan) was dissolved in the serum-free medium prior to use and exposed to cells at 37°C for desired time.

Survival study

Cell survival test was performed according to Terasima *et al.* (1972) with minor modifications. For colony formation assays, HeLa cells were in logarithmic growth phase. The cells were exposed to mutagens for desired time and then washed three times with PBS. Single-cell suspensions were prepared with 0.05% trypsin-EDTA. For experiments, 300 cells were plated per 60 mm diameter tissue culture dishes (Corning, U.S.A.). The plated cells were incubated for 14 days in growth medium and then fixed 2 times with Carnoy's solution. Colonies were stained with 4% Giemsa and then counted.

Lectin Cytochemistry

The changes of the glycoconjugates on plasma membrane were detected by lectin-cytochemistry according to the technique of Lundh *et al.* (1989) with minor modifications. The glycoconjugates on plasma membrane were detected with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.) using 0.05% diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, U.S.A.) as the chromogen, and all biotinylated lectins were obtained from Vector Laboratories (Burlingame, CA, USA). The lectin conjugates were diluted in tris-buffered saline (TBS), pH 7.4, containing 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. The exponentially growing cells were seeded onto cover glass, placed in petridishes and treated with mutagens for desired time, and then washed three times with PBS, and then fixed in fresh cold acetone at −20°C. The cells were then washed with PBS. Endogenous peroxidase activity was extinguished with a solution of 0.3% H_2O_2 in methanol for 30 min at room temperature, and then washed with PBS. To diminish non-specific binding of proteins, 5% normal rabbit serum and 2% bovine serum albumin in PBS were applied for 20 min at room temperature. The biotinylated lectin was applied to the cells for overnight at 4°C, and then washed three times with PBS. To demonstrate the biotinylated lectins, an avidin-biotin horseradish peroxidase complex was applied for 1 hour at room temperature, and then washed three times with PBS. The peroxidase activity was visualized using a solution of 1% H_2O_2 and 10% diaminobenzidine in TBS.

Results

Effects of pretreatment with low dose of UV, EMS, and BLM on survival in HeLa cells

Fig. 1 shows the effect of pre-exposure to 1 or 3 J/m² UV on survival of cells exposed to 5 J/m² UV. The percentage of survival of cells exposed to 5 J/m² (74.4±7.0) is higher

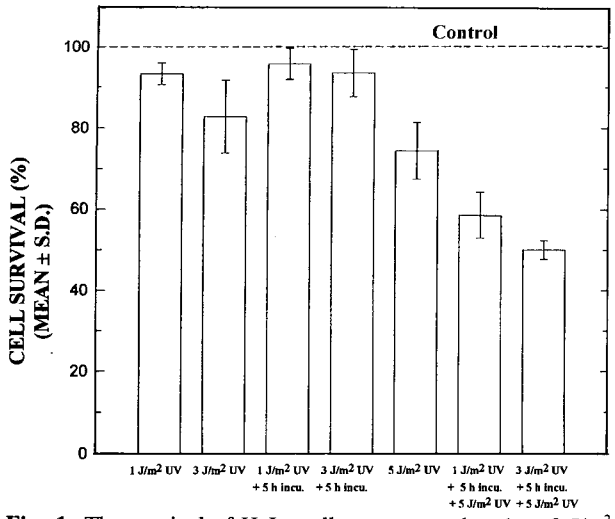

Fig. 1. The survival of HeLa cells pre-exposed to 1 or 3 J/m^2 UV following exposed to 5 J/m^2 UV. incu.; incubation.

than that of cells pre-exposed to 1 J/m^2 UV following exposed to 5 J/m^2 UV (58.5±5.0), and is higher than that of cells pre-exposed to 3 J/m^2 UV following exposed to 5 J/m^2 UV (50.0±2.0). Thus, the acquired resistance to UV is not shown in HeLa cells. The effect of pre-treatment with 1 mM EMS on survival of cells post-treated with 10 mM EMS is shown in Fig. 2. The percentage of survival of cells treated with 10 mM EMS (65.2±3.0) is lower than that of cells pre-treated with 1 mM EMS following treated with 10 mM EMS (73.2±3.0). That is, it is shown that the acquired resistance to EMS present in HeLa cells. Fig. 3 shows that the effect of pretreatment with 1 or 2 μg/ml BLM on survival of cells treated to 5 μg/ml BLM. The percentage of

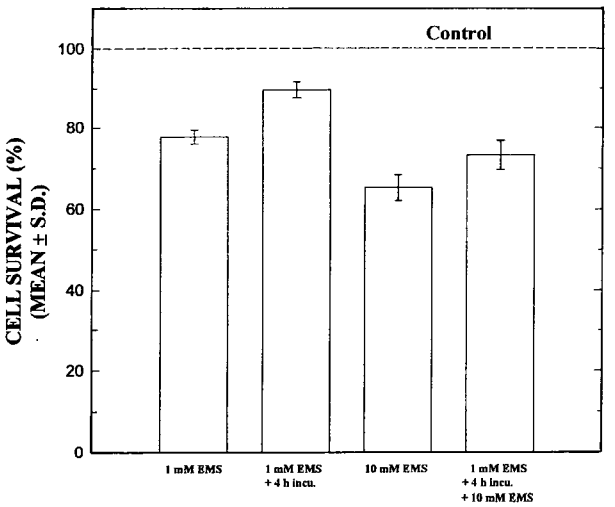

Fig. 2. The survival of HeLa cells pretreated with 1 mM EMS following treated with 10 mM EMS.

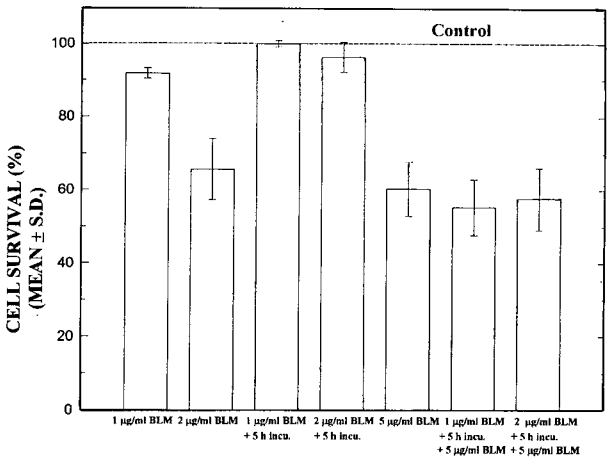

Fig. 3. The survival of HeLa cells pretreated with 1 or 2 μg/ml BLM following treated with 5 μg/ml BLM.

survival of cells treated with 5 μg/ml BLM (60.3±7.0) is similar to that of cells pretreated with 1 μg/ml BLM following treated with 5 μg/ml BLM (55.3±7.0), and similar to that of cells pretreated with 2 μg/ml BLM following treated with 5 μg/ml BLM (57.6±8.0). Thus, the acquired resistance to BLM is not shown in HeLa cells.

Effects of pretreatment with low dose of UV, EMS, and BLM on glycoconjugates in HeLa cells

Table 1 represents the origin, abbreviation, and sugar specificity of the biotinylated lectins used. The pattern of lectin binding in HeLa cells pre-exposed to 1 or 3 J/m^2 UV and subsequently exposed to 5 J/m^2 UV is shown in Table 2. All kinds of lectin staining intensity of cells pre-exposed to 1 or 3 J/m^2 UV and subsequently exposed to 5 J/m^2 UV was similar to that of cells treated with 5 J/m^2 UV. Table 3 represents the pattern of lectin binding in HeLa cells pretreated with 1 mM EMS and subsequently treated with 10 mM EMS. Wheat germ agglutinin staining intensity of cells pretreated with 1 mM EMS and subsequently treated with 10 mM EMS was stronger than that of cells treated with 10 mM EMS alone. But, succinylated wheat germ agglutinin staining intensity of cells pretreated with 1 mM EMS following treatment with 10 mM EMS was similar to that of cells treated with 10 mM EMS alone. Thus, the glycoconjugates containing sialic acid of plasma membrane in cells pretreated with 1 mM EMS and subsequently treated with 10 mM EMS was expressed more than that of cells treated with 10 mM EMS alone.

Table 4 shows the pattern of lectin binding in HeLa cells pretreated with 1 or 2 μg/ml BLM and subsequently treated

Table 1. Origin, abbreviation, and sugar specificity of the biotinylated lectins used

Lectin origin	Abbreviation	Lectin concentration (μg/ml)	Sugar specificity*
Triticum vulgaris	WGA	10	GlcNAc>Neu-5-Ac
Triticum vulgaris	sWGA	10	GlcNAc
Canavalia ensiformis	ConA	10	α-D-Man
Dolichos biflorus	DBA	10	GalNAc-α-1,3-GalNAc
Arachis hypogaea	PNA	20	galactosyl-β-1,3-GalNAc
Glycine max	SBA	20	D-GalNAc
Griffonia simplicifolia	GSLI	20	α-D-GalNAc & α-Gal
Ulex europaeus-I	UEAI	10	α-L-Fucose

WGA; Wheat germ agglutinin, sWGA; succinylated Wheat germ agglutinin, ConA; Concanavalin A, DBA; Dolichos biflorus agglutinin, PNA; Peanut agglutinin, SBA; Soybean agglutinin, GSLI; Griffonia simplicifolia Lectin I, UEAI; Ulex europaeus agglutinin I, GlcNAc; N-Acetyl-D-glucosamine, Man; Mannose, GalNAc; N-Aetyl-D-galatosamine, Neu-5-Ac; N-Acetyl neuraminic acid or sialic acid, α-Gal; α-galactose. *According to Leathem and Atkins (1983) & Goldstein and Poretz (1986).

Table 2. Lectin-binding pattern in HeLa cells pre-exposed to 1 or 3 J/m² UV and subsequently exposed to 5 J/m² UV

	Control	1 J/m² UV	3 J/m² UV	1 J/m² UV +5 h incu.	3 J/m² UV +5 h incu.	5 J/m² UV	1 J/m² UV +5 h incu. +5 J/m² UV	3 J/m² UV +5 h incu. +5 J/m² UV
WGA	++	++	++	++	++	++	++	++
sWGA	+	+	+	+	+	+	+	+
ConA	++	++	++	++	++	++	++	++
DBA	+	+	+	+	+	+	+	+
PNA	+	+	+	+	+	+	+	+
SBA	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GSLI	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
UEAI	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)

Staining intensities are given in arbitrary units; (+), very weak staining; +, weak staining; ++, moderate staining; +++, strong staining. incu.; incubation.

Table 3. Lectin-binding pattern in HeLa cells pretreated with 1 mM EMS and subsequently treated with 10 mM EMS

	Control	1 mM EMS	1 mM EMS +4 h incu.	10 mM EMS	1 mM EMS +4 h incu. +10 mM EMS
WGA	++	++	++	++	++
sWGA	+	+	+	+	+
ConA	++	++	++	++	++
DBA	+	+	+	+	+
PNA	+	+	+	+	+
SBA	(+)	(+)	(+)	(+)	(+)
GSLI	(+)	(+)	(+)	(+)	(+)
UEAI	(+)	(+)	(+)	(+)	(+)

Table 4. Lectin-binding pattern in HeLa cells pretreated with 1 or 2 μg/ml BLM and subsequently treated with 5 μg/ml BLM

	Control	1 μg/ml BLM	2 μg/ml BLM	1 μg/ml BLM +5 h incu.	2 μg/ml BLM +5 h incu.	5 μg/ml BLM	1 μg/ml BLM +5 h incu. +5 μg/ml BLM	2 μg/ml BLM +5 h incu. +5 μg/ml BLM
WGA	++	++	++	++	++	++	++	++
sWGA	+	+	+	+	+	+	+	+
ConA	++	++	++	++	++	++	++	++
DBA	+	+	+	+	+	+	+	+
PNA	+	+	+	+	+	+	+	+
SBA	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
GSLI	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
UEAI	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)

with 5 μg/ml BLM. All kinds of lectin staining intensity of cells pretreated with 1 or 2 μg/ml BLM and subsequently treated with 5 μg/ml BLM was similar to that of cells treated with 5 μg/ml BLM, respectively.

Discussion

The most consistent alteration found in multidrug resistance cell lines is an increased expression of a high molecular weight cell surface glycoprotein (P-gp) and the concomitant decrease in accumulation and retension of cytotoxic drugs (Riordan and Ling, 1985; Gottesman and Pastan, 1993). Such multidrug resistance is known to be conferred by at least two proteins, the Mr 170 kDa P-glycoprotein (P-170, encoded by the MDR1 gene) and the more recently identified 190 kDa multidrug resistance associated protein (Cole *et al.*, 1994). Overexpression of the Mr 170 kDa drug ATP-driven efflux pump called P-170 is responsible for the resistance of many multidrug resistant cell lines and some human tumors (Volm *et al.*, 1989; Cole *et al.*, 1994) to various hydrophobic (Endicott and Ling, 1989; Gottesman and Pastan., 1993) or positively charged cytotoxic drugs (Ford and Hait, 1990). And also, P-gp is composed of hydrophobic membrane-bound regions, hydrophilic N-terminal and nucelotide-binding regions, and sites for glycosylation and phosphorylation (Ford and Hait, 1990). Cells selected for resistance with one drug display significantly cross-resistance to the other drugs (Ford and Hait, 1990).

On the other hand, up to now the data on tumor models with inherent and acquired resistance *in vivo* are sparse and it is at present unclear, whether the overexpression of P-glycoprotein is solely responsible for the resistance of these tumors or not (Volm *et al.*, 1992). Thus, P-170 is not sufficient to explain the multidrug resistance satisfactorily (Efferth and Volm, 1993). And a wide variety of additional phenotypic properties that are unrelated to the overexpression of P-170 have also described (Huot *et al.*, 1991; Bhushan *et al.*, 1992). There is report that the expression of γ-glutamyltranspeptidase and P-170 is related with the general adaptive response to the harsh external condition (Kim *et al.*, 1991). And overexpression of the P-gp is recognized by the cell as an adaptive response to toxicity and the Fos protein could play a central role in this resistance, because this gene product activates the transcription of other genes (Bhushan *et al.*, 1992). Increased cellular multidrug resistance protein levels are associated with increased reduced glutathione (GSH) S-conjugate carrier (GS-X pump) activity in isolated plasma membrane (Jedlitschky *et al.*, 1994; Müller *et al*, 1994).

The UV response in HeLa S_3 cells is initiated at or near the plasma membrane, involving transcription factor AP-1, composed of jun and Fos proteins rather than the nucleus (Devary *et al.*, 1992). And, an elevation in *c-fos* could be functionally linked to the elevation of additional gene products, exactly in the manner observed for classic MDR (Bhushan *et al.*, 1992). The UV response may be elicited by oxidative stress, because it is inhibited by elevation of intracellular glutathione (Devary *et al.*, 1992). Transcription of both c-jun and c-fos is rapidly induced by exposure to UV-C (40 J/m^2) and other DNA-damaging agents (12-O-tetradecanoylphorbol-13-acetate), whereas transcription of other family members is not affected, at least not in HeLa S_3 cells (Devary *et al.*, 1991). And Bhushan *et al.* (1992) suggested that fos/jun transcriptional control element may participate in the regulation of P-gp expression. But, in our experiments, HeLa cells exposed to 1 or 3 J/m^2 UV-C did not show the changes the glycoconjugates of plasma membrane.

On the other hand, Chou and Kessel (1981) showed that Adriamycin treatment led to enhanced glycosylation of tumor cell membranes. And, Chinese hamster lung cells selected for resistance to dactinomycin, daunorubicin, or vincristine have increased amounts of 150 kDa glycoprotein and altered gangliosides in the membrane (Biedler *et al.*, 1981). Dickstein *et al.* (1995) showed that ricin A binding to glycoprotein was involved in resistance to adriamycin. Overexpression of 95 kDa membrane protein (N-linked sialoglycoprotein) has been reported in MDR breast cancer cell line (MCF-7/AdrVp) and MDR small cell lung cancer line (NCI-H1688) (Doyle *et al.*, 1995). Considering other's and our results, it is suggested that WGA-binding glycoconjugates of plasma membrane in HeLa cells are involved in acquired resistance to EMS. However, further studies will be necessary to elucidate whether WGA-binding glycoconjugates of plasma membrane are involved directly (as drug transporter) or indirectly (as signal trigger of resistance gene expression) for the acquired resistance to EMS in HeLa cells.

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HeLa세포에서 원형질 막의 glycoconjugate에 대한 수종 돌연변이원의 전처리 효과

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본 연구는 돌연변이원인 자외선과 **ethyl methanesulfonate(EMS)** 그리고 **bleomycin(BLM)**을 전처리한 **HeLa** 세포에서 세포막에 존재하는 **glycoconjugates**에 미치는 이들 돌연변이원의 효과를 밝히기 위하여 세포생존 실험법과 **Lectin cytochemistry**법으로 조사하였다. **1 mM EMS**를 전처리한 후 **10 mM EMS**를 처리한 군의 세포생존율은 **10 mM EMS**를 단독처리한 군의 세포생존율 보다 더 높았다. 그리고 **10 mM EMS** 단독처리군 보다 **1 mM EMS**를 전처리 한 후 **10 mM EMS**를 처리한 군에서 **Wheat germ agglutinin (WGA)**은 더욱 강한 양성반응을 보였다. 반면에 **succinylated wheat germ agglutinin (sWGA)**는 **10 mM EMS** 단독처리군과 **1 mM EMS**를 전처리한 후 **10 mM EMS**를 처리한 군에서 차이가 없었다. 이상의 결과들을 종합해 볼 때, **HeLa** 세포에서 **EMS**에 대한 획득된 저항성은 다내약제성 또는 적응반응과 관련되는 원형질막의 **sialic acid**를 포함한 **glycoconjugates**와 관계 될 것으로 추측된다.