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CHARACTERIZATION OF MULLERIAN INHIBITING SUBSTANCE BINDING ON CERVICAL CARCINOMA CELLS DEMONSTRATED BY IMMUNOCYTOCHEMISTRY

Keywords: Müllerian inhibiting substance; immunocytochemistry; human cervical carcinoma CaSki cell

ABSTRACT. Müllerian inhibiting substance (MIS) is a glycoprotein released from Sertoli cells or follicular cells of gonads, responsible for the regression of Müllerian ducts and/or Müllerian-derived tumor cells. Binding of MIS to target cells is essential for initiating regression. A human cervical carcinoma CaSki cell was examined by quantitative immunocytochemistry detected by anti-avian MIS antibody for MIS binding ability. Various treatments of WGA-peroxidase conjugate, enzyme digestion, sodium periodate or exogenous estrogen before antibody recognition were performed. It was found that the WGA partially blocked MIS binding to CaSki cell surfaces. Protease digestion of CaSki cell surfaces prior to addition of MIS or an anticervical carcinoma monoclonal antibody 1H10 (MAb 1H10), blocked the binding of MIS but not MAb 1H10 to cell surfaces. Sodium periodate and overnight exposure of CaSki cells to estrogen or diethylstilbestrol before or after fixation of the cells, did not influence MIS binding ability *in vitro*. MIS binding was higher on avian Müllerian duct compared with MIS binding to CaSki cells by quantitative immuno-gold labeling analysis. MAb 1H10 immuno-gold complexes binding to CaSki cells was also obtained and compared with MIS immuno-gold bindings. MIS binding site could be a polypeptide which survived sodium periodate treatment. The 'critical window' period, in which developing Müllerian ducts respond to exogenous estrogen protection from MIS regression, is possibly lost in CaSki cell.

Introduction

Müllerian inhibiting substance (MIS) has been identified as a glycoprotein secreted by embryonic Sertoli cells in male gonads (Tran *et al.*, 1987; Wang *et al.*, 1990) and in gran-

ulosa cells in female gonads (Takahashi *et al.*, 1986). MIS is responsible for the regression of Müllerian ducts in male animals. Similarly in female chicks, the right Müllerian duct also regresses upon MIS stimulation; the left Müllerian duct, however, does not regress and develops into a functional reproductive tract (Josso and Picard, 1986). Our previous work indicated that MIS binding is high on the apical surfaces of epithelial cells of the right and left Müllerian ducts of day 7 1/2 and day 13 of gestation (Wang, 1989). MIS can also be detected on Müllerian ducts of new born chicks, however, it is not present on Wolffian ducts in either female or male chicks at developmental stages (Wang, 1989).

Cervical carcinoma of Müllerian origin (Wang and Teng, 1989) is a common lethal

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malignancy in Taiwan (Yeh, 1985). Efforts have been made by inducing and characterizing a monoclonal antibody, MAb 1H10, against CaSki cell-specific epitope (Roffler *et al.*, 1991a; 1991b; Wang *et al.*, 1992). It has been demonstrated that MIS inhibits the growth of ovarian carcinoma cells *in vitro* (Donahoe *et al.*, 1984; Fuller *et al.*, 1985; Chin *et al.*, 1991) as well as *in vivo* (Donahoe *et al.*, 1984). Growth of other Müllerian-derived tumor cells have also been detected to be inhibited by MIS (Donahoe *et al.*, 1984; Fuller *et al.*, 1984). For growth inhibition of the target cells, binding of MIS to the cell surfaces is the first step for MIS-induced regression of Müllerian ducts through a receptor-mediated mechanism (Wang, 1989). The possible existence of MIS binding sites (or receptors) has been examined on Müllerian ducts *in vivo* (Wang *et al.*, 1990) as well as on A431 and other cervical carcinoma cells of Müllerian origin after treatment with a synthetic estrogenic hormone diethylstilbestrol (DES) (Wang and Teng, 1989). The appearance of MIS binding sites may correlate with MIS-induced regression (Wang, 1989).

The fact that avian MIS has been purified by wheat germ agglutinin (WGA) affinity chromatography indicates that MIS is a glycoprotein rich in N-acetyl-galactosamine (Teng *et al.*, 1987). Teng and Teng (1985) have proved that steroid hormones alone do not affect the regression of chick oviducts. Other steroid hormones, testosterone and FSH, have been shown to have no influence on the secretion of MIS from Sertoli cells in primary culture (MacLaughlin *et al.*, 1983). However, treatment with exogenous estrogen or DES during the early stages of embryonic development prevents the regression of Müllerian ducts and causes feminization of gonads (MacLaughlin *et al.*, 1983; Hutson *et al.*, 1982; Newbold *et al.*, 1984; Wang *et al.*, 1990). E₂ or DES pre-treatment blocked binding of MIS to target cell surfaces and provided protection from MIS-induced regression (MacLaughlin *et al.*, 1983; Wang *et al.*, 1990).

In this study, the MIS binding to cervical carcinoma CaSki cells, compared to the regressing and growing Müllerian ducts was examined by immunocytochemical analysis. The effects of pre-treatment with proteolytic enzymes, steroid hormones, and sodium per-

iodate as well as WGA competition on MIS binding to the cell surfaces were examined and quantitative comparison to MAb 1H10 binding on CaSki cell was also performed.

Materials and Methods

Cell lines, MIS and Müllerian ducts

CaSki cell line was obtained from ATCC (American Type Culture Collection). The cells were maintained in RPMI 1640 culture medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 5 mM benzamidine as described (Wang *et al.*, 1992). The cells were seeded on Thermanox round plastic coverslips (Nunc, Kamstrup, Denmark) in 24-well plates. Purified avian MIS was obtained as described previously (Teng, 1987). Right and left Müllerian ducts were collected separately from chick embryos at 13 days of gestation (Wang, 1989).

E₂/DES or MIS treatment

β-estradiol (E₂, Sigma, E-8875) or diethylstilbestrol (DES, Sigma, D-4628) were dissolved at a concentration of 10 mg/ml in 70% alcohol (Wang *et al.*, 1991). Unfixed or fixed CaSki cells were incubated in 10 ml culture medium with two drops of 50 ng/ml of E₂ or DES overnight at 37°C. For MIS treatment, the cell samples were incubated with 0.25–0.5 µg/ml affinity purified avian MIS (Takahashi *et al.*, 1986; Teng *et al.*, 1987) for 10 min at room temperature followed with primary antibody incubation.

Enzyme digestions

Fixed or unfixed CaSki cells on plastic coverslips were reacted with protease or sodium periodate at 37°C for 1 hr at the following concentrations: Trypsin (0.05%) in 40 mM Tris-HCl buffer, pH 8.1 with 50 mM CaCl₂; protease (0.05%) in 40 mM Tris-HCl, pH 7.8; or saturate sodium periodate (35% in distilled water) incubation for 20 min at room temperature. After various treatments, cell samples were washed and incubated with anti-MIS serum (Wang and Teng, 1989) or MAb 1H10 (Roffler *et al.*, 1991b) for 1 hr at room temperature. Antibody binding to treated CaSki cells was detected by subsequent incubation with anti-rabbit or anti-mouse IgG conjugated with colloidal gold particles.

WGA binding and immunoelectron microscopy

WGA-peroxidase (PO) conjugates were incubated with fixed CaSki cells for 1 hr at room temperature (Wang *et al.*, 1990) and then examined for their ability to block the binding of anti-MIS antibody or MAb 1H10 to cells by pre-embedding immunostaining (Wang *et al.*, 1992) and flat embedding (Chang and Wang, 1993) techniques. After washing three times, cells were incubated with anti-MIS antibody or MAb 1H10, washed, and then incubated with immuno-gold (10 nm in diameter) conjugates. WGA-PO conjugate binding was visualized by reaction with diaminobenzidine (DAB, 0.5 mg/ml) and hydrogen peroxide (0.04%) in 0.05 M Tris-HCl buffer, pH 7.6, for 15 min. Control groups were incubated in a culture medium contains no primary antibody (anti-MIS antibody or MAb 1H10).

Quantitative assay of antibody bindings

Anti-MIS antibody or MAb 1H10 binding to cells was quantified by using a Molecular Dynamics (Sunnyvale, California, USA) computing densitometer to measure the intensity of the second antibody-peroxidase conjugate catalyzed reaction product (Wang *et al.*, 1992). The numbers of MIS- or 1H10-gold particles on the surface of CaSki cells after each treatment and MIS-gold particles on apical surfaces of Müllerian ducts were counted and expressed as the average number of gold particles per μm at the magnification of $\times 10,000$. 15–25 micrographs of each sample were counted.

Results

In order to investigate the distribution of MIS bound to the surface of CaSki cervical carcinoma cell, fixed or unfixed cells grown on plastic coverslips were incubated with anti-avian MIS serum followed by goat anti-rabbit IgG conjugated with peroxidase. CaSki cells with or without pre-treatment of exogenous MIS were positively stained for MIS binding. Neither addition of exogenous MIS nor exogenous estrogen significantly altered the intensity of MIS binding (Fig. 1a, lanes 1–4 in Fig. 4). A negative control, in which anti-MIS serum was omitted, did not stain for peroxidase under light microscopic

observation (Fig. 1b, lane 5 in Fig. 4). Under the electron microscope, MIS was found to bind to the cell surface and in vesicles attached to the cell membrane (Fig. 1c). Anti-MIS antibody-gold particles as well as positive PO deposits were observed on the surface of CaSki cells when cells were first reacted with WGA-PO (Fig. 2a, lane 7 in Fig. 4). Control cells which were incubated with WGA-PO but not anti-MIS serum showed negative MIS-gold bindings (Fig. 2b).

The results of characterization of MIS binding compared with MAb 1H10 binding to CaSki cells by immunoelectron microscopy are summarized in Table 1. Cells were also pre-treated with various enzymes before incubation with anti-MIS antiserum to examine their effects on MIS-Ab bindings (Table 1). MAb 1H10 binding to CaSki cells has been previously detected by immuno-PO (Wang *et al.*, 1992) and immuno-gold tracings. The intensity of MIS binding is less than MAb 1H10 binding on CaSki cell surfaces (Lane 6 in Fig. 4). Treatment of CaSki cells with sodium periodate reduced MAb 1H10-gold but not MIS-gold binding (Lane 8 in Fig. 4). Trypsin or protease digestion of CaSki cells reduced the MIS antiserum staining (Lane 13 in Fig. 4), whereas 1H10 binding was not affected (Lane 14 in Fig. 4). Previous data have shown that chondroitinase treatment of CaSki cells eliminated 1H10 binding (Wang *et al.*, 1992). WGA interfered with normal MIS binding, but E_2 treatments before or after fixation of the cells did not affect the intensity of MIS binding (Table 1).

MIS immuno-gold bound to the apical surfaces of the left (Fig. 3a) and right Müllerian ducts (Fig. 3b) were counted respectively. There was an average of 2.67 MIS-gold particles/ μm of the cell membrane at magnification of $\times 10,000$. The right Müllerian ducts are regressing embryonic organs and had 20.3 MIS-gold particles/ μm , whereas the left Müllerian ducts are growing organs and bound 7.02 MIS-gold particles/ μm (Lanes, 9, 10 in Fig. 4). An average of 4.88 MAb 1H10-gold bound on CaSki cells (Lane 6 in Fig. 4). No MIS binding was observed on embryonic Wolffian ducts (Fig. 3c, lane 11 in Fig. 4). Other control groups omitting primary antibody incubation also showed no MIS immunolabeling (Table 1 and Fig. 4).

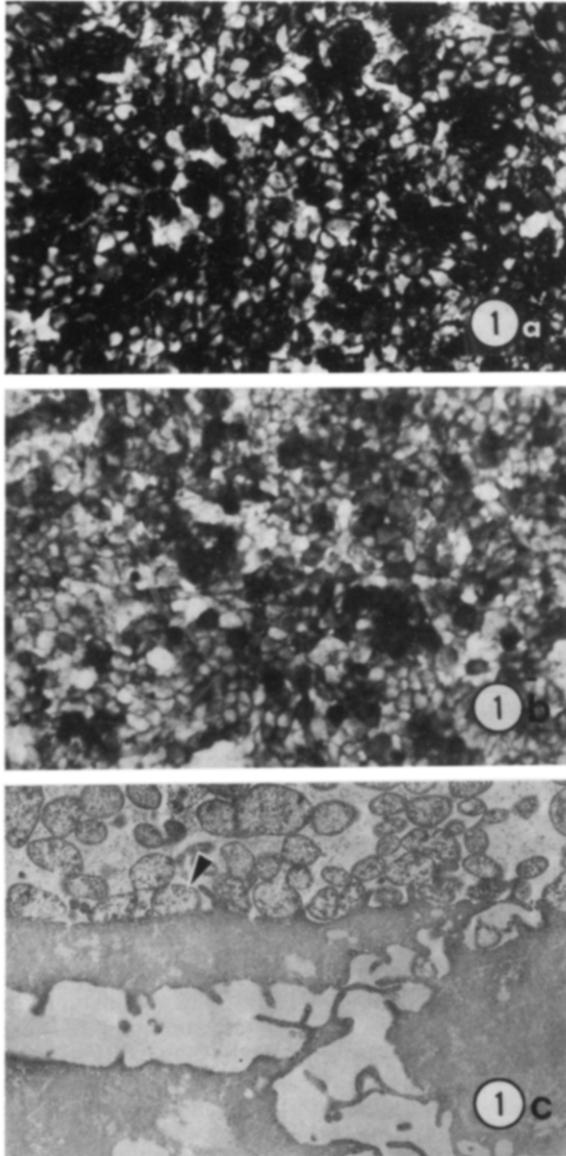


Fig. 1. CaSki cells grown on plastic coverslips were examined by anti-MIS serum and immunoperoxidase (PO) labelings after fixation. Positive MIS-PO bindings were shown as dense deposits on surfaces of CaSki cells (Fig. 1a, $\times 750$). The control group omitting primary antibody incubation was negative of PO reaction (Fig. 1b, $\times 750$). Positive MIS-PO deposits were found on the cell surface and in the vesicles (arrowhead) near the cell membrane when examined under the electron microscope (Fig. 1c, $\times 9,300$).

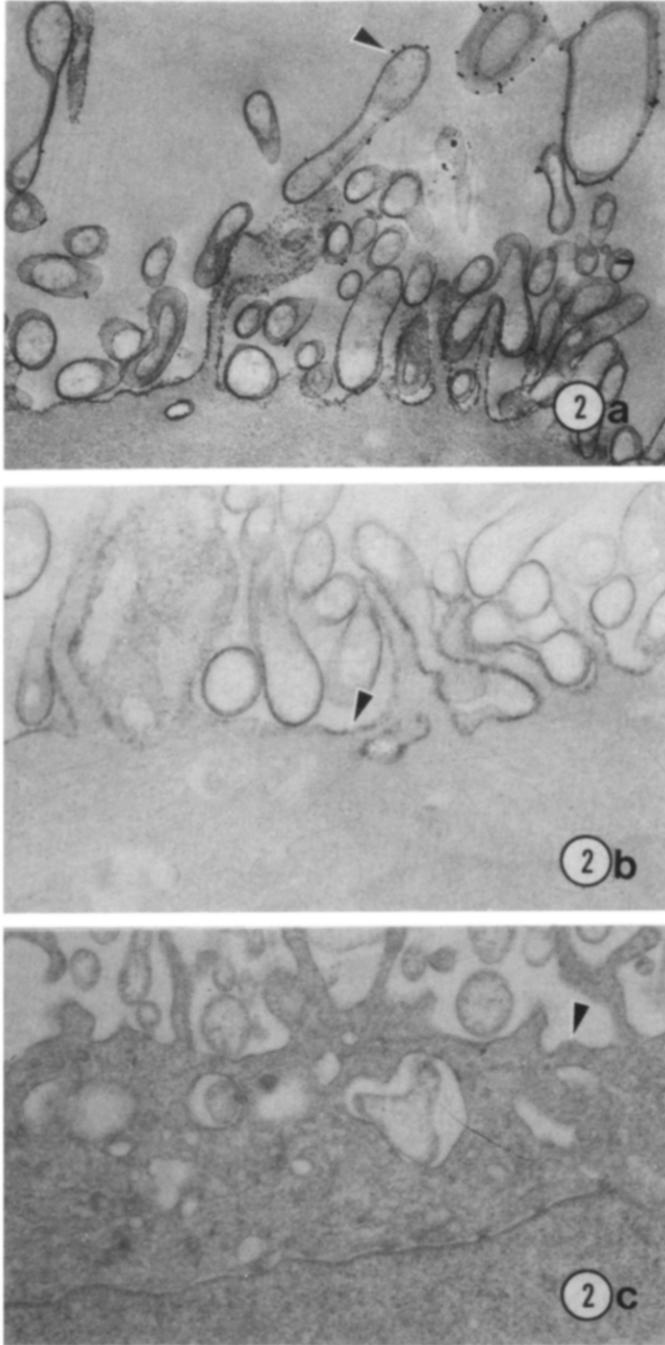


Fig. 2. WGA-peroxidase and MIS immuno-gold labelings on CaSki cell surfaces. CaSki cells on coverslips were labeled with anti-MIS serum and immuno-gold complexes (arrowhead) on the cell surface after WGA-PO binding (Fig. 2a, $\times 15,500$). Cells labeled with WGA-PO, but without primary anti-MIS antibody treatment showed positive peroxidase reaction but no immuno-gold binding (arrowhead) (Fig. 2b, $\times 27,000$). Negative MIS-gold bindings (arrowhead) was observed on the cell surface after trypsin or protease digestion of cell samples on coverslips (Fig. 2c, $\times 16,700$).

Table 1. Characterization of MIS binding to CaSki cells, Müllerian and Wolffian ducts (as indicated) in comparison with MAb 1H10 binding

Treatment	1st Ab	2nd Ab or WGA	PO	Gold
Non	MIS	Ab-G	/	+
Non	MIS	Ab-PO	++	/
MIS	MIS	Ab-G	/	+
E ₂ /unfixed	MIS	Ab-G	/	+
E ₂ /fixed	MIS	Ab-G	/	+
Non	Non	Ab-G	-	/
Non	1H10	Ab-G	/	++
WGA-PO	MIS	Ab-G	+	+
WGA-PO/R-Md	MIS	Ab-G	+	+++
WGA-PO/L-Md	MIS	Ab-G	+	+
Non/Wd	MIS	Ab-G	/	-
WGA-PO	MIS	Ab-G	+	+
Sod periodate	non	WGA-PO	-	/
Sod periodate	MIS	Ab-G	/	+
Trypsin	MIS	Ab-G	/	-
Protease	MIS	Ab-G	/	-
Trypsin	1H10	Ab-G	/	++
Protease	1H10	Ab-G	/	++

Ab: Antibody; PO: Immuno-peroxidase; G: Immuno-gold; R-Md: Right Müllerian duct; L-Md: Left Müllerian duct; Wd: Wolffian duct; '-': Negative reaction; '/': Not available; '+': Positive reaction; '++': Strong positive; and '+++': Very strong positive reaction.

WGA and sodium periodate-PO treatments did not significantly block MIS and 1H10 binding on the right Müllerian duct nor on the left Müllerian duct (Lanes 9, 10 in Fig. 4). The quantitative data of MIS- and 1H10-gold particles after various treatments of WGA or enzymes are shown in Figure 4 and summarized in Table 1.

Discussion

The present study has examined the binding profiles of MIS on CaSki cervical carcinoma cells pretreated with exogenous MIS, estrogen, WGA, enzyme digestions or sodium periodate. The results were obtained by immunoelectron microscopy and quantitative immuno-gold analysis. MIS immuno-gold binding on chick Müllerian ducts *in vivo* was compared with MIS immuno-gold binding on CaSki cervical carcinoma cells. MAb 1H10, which recognizes a mucopolysaccharide binding site, was also examined for its binding on CaSki carcinoma cells after

similar pre-treatments and compared to MIS binding.

Exogenous avian MIS was added to the culture medium of CaSki cells for 10 min at 37°C in order to determine whether additional MIS binding sites exist on these cells. There was no significant increase in MIS binding after addition of exogenous MIS, indicating that MIS binding sites on CaSki cells are already saturated. CaSki carcinoma cells showed no altered fine structural changes after acute MIS treatment.

MIS fulfills its function by binding to the cell surface of its target cells in a quantitative relationship to the degree of regression of Müllerian-derived carcinoma cells (Fuller *et al.*, 1984 & 1985; Coughlin *et al.*, 1987) and avian Müllerian ducts before 15 day of gestation (Behringer *et al.*, 1990; Wang, 1989; Wang and Teng, 1989). The amount of MIS binding on developing Müllerian ducts was 4-5 fold greater than on CaSki cells and other Müllerian-derived tumor cells (Wang and Teng, 1989). The amounts of MIS-gold that

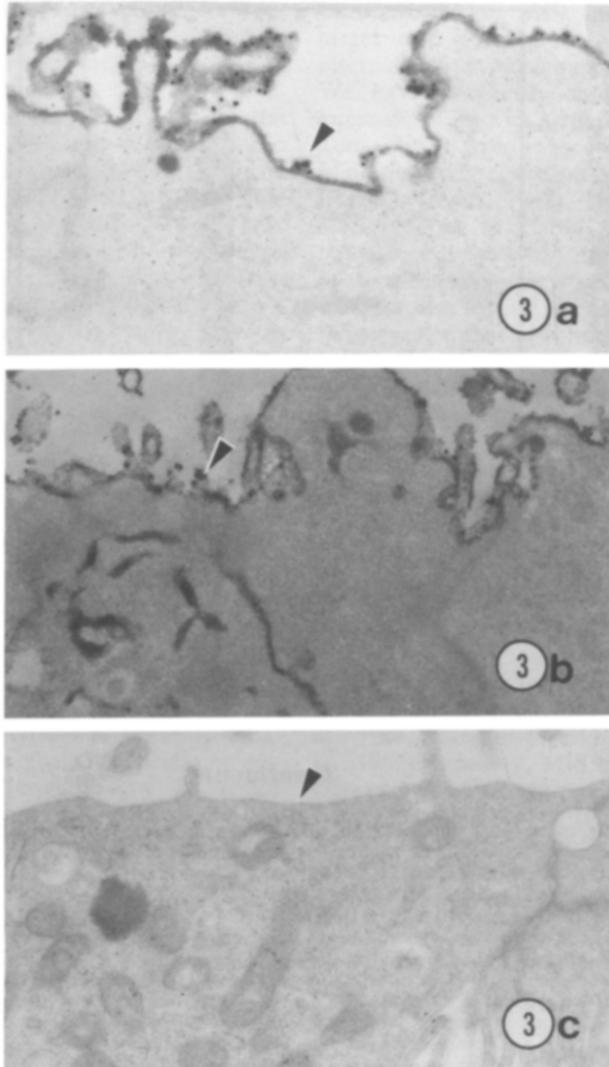


Fig. 3. WGA-peroxidase and MIS immuno-gold labelings on chick Müllerian ducts. WGA-PO binding before MIS antibody recognition did not interfere the MIS immuno-gold labeling (arrowhead) on the left Müllerian duct (Fig. 3a, $\times 31,500$). WGA-PO labeling before MIS antibody addition did not interfere with MIS immuno-gold labeling (arrowhead) on the right Müllerian duct (Fig. 3b, $\times 18,300$). However, the right Müllerian duct bound more MIS-gold particles than did the left duct. A control of Wolffian duct cells were negative for MIS-gold (arrowhead) immuno-labeling (Fig. 3c, $\times 18,000$).

bound on CaSki cells before or after addition of exogenous MIS were similar to the amount of MIS-gold that bound on the growing left Müllerian ducts. The consistent presence of

MIS binding to CaSki cells strongly indicates the presence of MIS binding sites.

MIS binding on fixed or unfixed CaSki carcinoma cells was not affected by exogen-

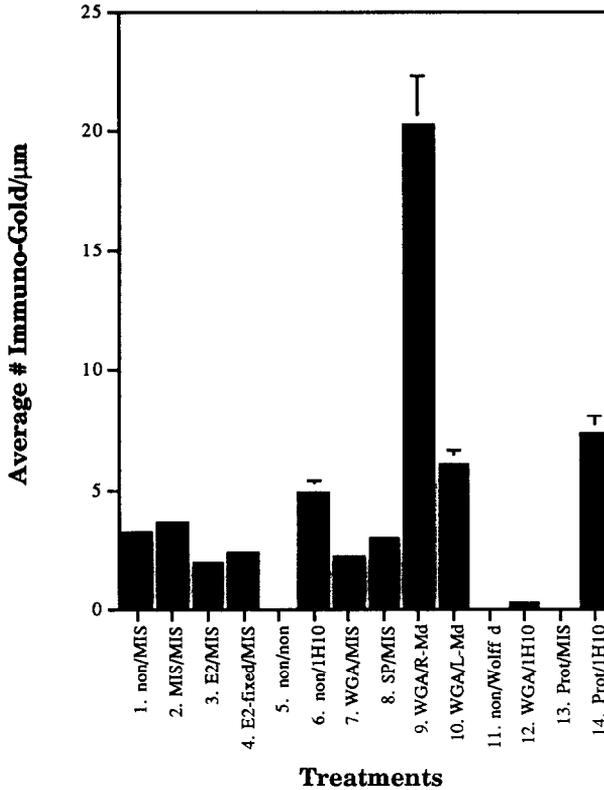


Fig. 4. Expression of MIS-gold on target cells. Quantitative data of MIS and 1H10 immuno-gold labelings after various treatments to CaSki cells and Müllerian ducts are shown as columns. Shown are results for MIS-gold labeling of CaSki cells without addition of exogenous MIS (lane 1); after addition of exogenous MIS (lane 2); with E₂ treatment of unfixed cells (lane 3) and E₂ treatment of fixed cells (lane 4). A control group in which primary anti-MIS serum was omitted (lane 5) showed negative MIS binding to CaSki cells. Similar to MIS-gold binding, MAb 1H10-gold labeling was revealed on CaSki cells (lane 6). After WGA-PO labeling (lane 7) or sodium periodate (SP, lane 8) treatment, MIS-gold still binds to CaSki cells. WGA-PO labeling did not interfere with MIS-gold binding on Müllerian ducts (lane 9, 10). MIS did not bind on Wolffian duct (lane 11). With previous WGA-PO binding, 1H10-gold labeling was reduced (lane 12). After protease digestion, no MIS-gold was found on CaSki cells (lane 13), whereas MAb 1H10 still bound after protease digestion of CaSki cells (lane 14).

ous E₂ or DES treatment. These results indicate that in CaSki cells, E₂ or DES cannot induce a down-regulation of MIS binding sites, which are responsible for MIS binding. MacLaughlin *et al.* (1983) also suggested that E₂ or DES cannot bind to MIS and prevent anti-MIS serum recognition of cell-bound MIS. However, previous studies indicated that exogenous treatment of DES was able to protect Müllerian ducts from MIS-induced

regression in chick embryos (Hutson *et al.*, Teng, 1987; Stoll *et al.*, 1982). DES was demonstrated to protect Müllerian ducts through a mechanism of preventing MIS binding (Wang *et al.*, 1990). Further results of a competitive ELISA technique indicated that MIS content is high in gonads after DES treatment (Teng, 1987). Exogenous DES cannot inhibit MIS secretion from gonads (Wang and Teng, 1989). Thus, E₂ or DES

may modulate the conformation of MIS binding molecules or receptor *in situ*, and then MIS can no longer bind on Müllerian ducts (Wang *et al.*, 1990).

The response of Müllerian ducts to the action of MIS has been shown to be limited to a 'critical window' period (Takahashi *et al.*, 1986; Taguchi *et al.*, 1984). MIS is able to induce regression of Müllerian ducts only between days 5–7.5 of gestation, even though MIS can still bind on Müllerian ducts after the 'critical window' period (Wang *et al.*, 1989). The binding of MIS to regressing or developing Müllerian ducts may induce programmed cell death during development (Price *et al.*, 1977). The results of treating CaSki cells with E₂ or DES suggest that a 'critical window' period is not present in CaSki carcinoma cells, although the effects of MIS on CaSki cell growth has not been yet determined.

WGA binds specifically to terminal monosaccharide, N-acetyl-galactosamine, present on glycan sub-groups on cell surfaces which may interfere with the binding of antibodies specific for polysaccharide epitopes but not polypeptide epitopes. Anti-MIS serum is specific for the polypeptide portion of the MIS molecules, so protease treatment can block anti-MIS antibody binding. The result that treatment of sodium periodate do not block MIS binding on CaSki cells, indicated that MIS and MIS binding molecules are not polysaccharide in nature. Sodium periodate and enzymes specific for different monosaccharides have been used to identify the terminal sugars recognized by MAb 1H10, showing that this antibody recognizes a mucopolysaccharide epitope (Wang *et al.*, 1992).

Growth-related polypeptide hormones or

factors, including MIS, induce response in target cells through a receptor-mediated internalization mechanism (Wang, 1989). We have previously shown that MIS is internalized into its target cells (Wang and Teng, 1989) with subsequent induction of Müllerian duct regression (Fuller *et al.*, 1984; Coughlin *et al.*, 1987). However, whether MIS binding to CaSki cells can induce regression of these cells through a receptor-mediated mechanism is still unknown.

According to our previous observations, we suggested that MIS binding site could be a protein or glycoprotein recognized by MIS on CaSki cells as well as on other Müllerian-derived tumor cells (Wang and Teng, 1989) and Müllerian ducts (Wang, 1989). Furthermore, MIS induced regression of Müllerian ducts could function only during a short period at early developmental stages within the 'critical window', which can be 'closed' by E₂ or DES treatment (Wang *et al.*, 1990) indicated by the binding ability of MIS (Wang, 1989). Since Müllerian-derived tumor cells have been demonstrated to be inhibited by MIS treatment (Wang, 1988; Wang *et al.*, 1990; Chin *et al.*, 1991; Fuller *et al.*, 1984; Wang and Teng, 1989; Hutson *et al.*, 1982), however, MIS binding on CaSki cells cannot be down-regulated by estrogen hormone treatment, it is suggested that a 'critical window' sensitive to MIS regression possibly does not exist in CaSki cells.

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