Dexamethasone and Medroxyprogesterone Acetate Elevate Nm23-H1 Metastasis Suppressor Gene Expression in Metastatic Human Breast Carcinoma Cells: New Uses for Old Compounds

Taoufik Ouatas, Douglas Halverson, and Patricia S. Steeg

Women’s Cancers Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892

ABSTRACT

Purpose: Long-term elevation of metastasis suppressor gene expression in micrometastases represents a novel therapeutic strategy for breast and other cancers. We searched for well-tolerated compounds that could elevate Nm23 metastasis suppressor expression in metastatic human breast cancer cell lines.

Experimental Design: MDA-MB-435 and MDA-MB-231 human breast carcinoma cells were treated with dexamethasone or medroxyprogesterone acetate (MPA) in cultures containing either charcoal-stripped serum or FCS. Aspects of nm23 expression and function were determined.

Results: Previous investigation of the nm23-h1 promoter suggested that glucocorticoids may contribute to the elevation of Nm23-H1 expression. Dexamethasone elevated Nm23-H1 and Nm23-H2 protein levels in two metastatic human breast carcinoma cell lines 2-3-fold over a 4-day time course when cultured in steroid-free culture medium, with high-dose inhibition, via a traditional transcriptional mechanism. Elevation of Nm23-H1 expression was not observed using FCS-containing culture medium, which contains endogenous levels of corticosteroids, limiting the potential in vivo use of dexamethasone. MPA was investigated as a glucocorticoid receptor agonist. MPA elevated breast carcinoma Nm23-H1 protein expression 3-fold over a 10 nM to 1 µM dose range when cultured in steroid-free or FCS-containing medium, with a shorter time course. Elevation of Nm23-H1 expression in the presence of endogenous corticosteroids found in FCS involved a distinct, glucocorticoid receptor-dependent, posttranscriptional mechanism of action. MPA had no effect on proliferation in vitro but reduced the soft agar colonization of metastatic breast cancer cell lines by ~50%.

Conclusions: MPA represents a first generation lead agent for the elevation of Nm23-H1 metastasis suppressor expression and the inhibition of metastatic colonization.

INTRODUCTION

 Interruption of the metastatic process may represent an important therapeutic target for breast and other cancers in the adjuvant setting. Metastasis suppressor gene expression constitutes a new approach to cancer therapy. Metastasis suppressor genes exhibit reduced expression levels and presumably protein function in highly metastatic tumor cells. Eight metastasis suppressor genes have been confirmed in transfection studies to date, nm23, M KK4, KAI-1, BRMS-1, KiSS-1, RhoGDI2, CRSP3, and VDUP-1; in each case, re-expression of the metastasis suppressor gene into a metastatic tumor cell line inhibited metastasis in vivo without a significant reduction in primary tumor size (reviewed Refs. 1, 2). Both in vitro and in vivo data indicate that metastasis suppressors inhibit metastatic colonization, the outgrowth of tumor cells at a foreign site via nonangiogenic mechanisms (2–7). Metastasis suppressors often use novel mechanisms of action, including the induction of differentiation, increased gap-junctional communication, and the modulation of specific signal transduction pathways (2). For the KAI-1 and nm23 metastasis suppressor genes, reduced expression rather than mutation was observed in aggressive human cancer cohorts (8, 9). From a cancer therapeutic perspective, a goal is the identification of well-tolerated compounds that could be delivered chronically and would elevate the metastasis suppressor expression of micrometastatic tumor cells.

The nm23 gene family was identified by its reduced expression in highly metastatic murine melanoma cell lines, as compared with related, tumorigenic but less metastatic cell lines (10) and now consists of eight family members (reviewed in Ref. 11). The metastasis suppressor activity of nm23 cDNAs has been demonstrated in multiple transfection experiments (7, 12–20). Among the human breast carcinoma cell lines investigated, transfection of nm23-h1 resulted in reduced colonization in soft agar, both unstimulated and when TGF-β2 was added to the cultures (7), and reduced invasion and motility to a variety of chemoattractants (14, 21–23). Nm23-H1 transfectants of the MDA-MB-435 breast carcinoma cell line exhibited morphological (ascus formation) and biosynthetic aspects of differentiation in three-dimensional culture (24); this finding is supported.

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1 To whom requests for reprints should be addressed, at Building 10, Room 2A33, NIH, Bethesda, MD 20892. Phone: (301) 402-2732; Fax (301) 402-8910; E-mail: steegp@mail.nih.gov.

2 The abbreviations used are: TGF-β, transforming growth factor β; AR, androgen receptor; CSS, charcoal-stripped serum; GR, glucocorticoid receptor; GRE, glucocorticoid response element; MMTV, mouse mammary tumor virus; CAT, chloramphenicol acetyltransferase; MPA, medroxyprogesterone acetate; PR, progesterone receptor.
by similar differentiation studies in neural cells (25–31). In most
but not all human breast tumor cohort studies, low Nm23
expression was significantly correlated with an aspect of aggres-
sive behavior (reviewed in Ref. 32). We hypothesize that ele-
vation of Nm23-H1 expression in micrometastatic tumor cells
from breast cancer patients whose primary tumors were low
Nm23-H1 expressing, may inhibit additional colonization and
invasion, with a clinical benefit.

Although several compounds have been reported to elevate
Nm23 expression in vitro, none appear to be good clinical
candidates. All trans-retinoic acid elevated Nm23-H1 expres-
sion in a human hepatocellular carcinoma cell line (33) and in
the metastatic MDA-MB-231 breast carcinoma cell line (unpub-
lished observation). However, the clinically used fenretinide
failed to elevate Nm23-H1 expression (unpublished data). Es-
tradiol increased Nm23-H1 expression in the nonmetastatic
MCF-7 and BT-474 human breast carcinoma cell lines (34).
Similarly, indomethacin elevated Nm23-H1 expression only in
less aggressive MCF-7 and MCF-12A cells but not in metastatic
MDA-MB-231 or MDA-MB-435 cell lines (35). γ-Linolenic
acid elevated Nm23-H1 expression weakly in human cell lines
(36). Previously noted that the DNA methylation inhibitor
5-azacytidine altered the DNA methylation pattern of a
CpG island in the nm23-H1 promoter and increased the
Nm23-H1 expression of metastatic breast carcinoma cell lines;
however, these findings were only infrequently repeatable for
the same nm23-H1 promoter CpG island isolated from human
breast carcinomas (37).

We recently identified a 2.1-kb fragment of the nm23-H1
promoter, which directed high versus low reporter gene expres-
sion when transiently transfected into a series of four human
breast carcinoma cell lines of varying in vivo metastatic poten-
tials (38). Restriction fragment deletion analysis identified a
248-bp region of the promoter as contributing to differential
expression, which contained three transcription factor binding
sites found in the MMTV-long terminal repeat (1) and other
mammary-specific gene promoters (Eis/MAF, CTF/NF1 half-
site, and ACACAAG enhancer). A pBT plasmid containing the
248-bp promoter fragment and an adjacent 195-bp fragment
containing both the transcription initiation site and two GREs
also mediated high versus low nm23-H1 reporter gene expres-
sion, and mutation of the mammary-specific sites established a
functional relationship. These sites, in a similar geographic
pattern, contribute to MMTV function: glucocorticoids bind to
the GR and the pair to the GRE, altering nucleosomal structure
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MATERIALS AND METHODS

**Cell Culture and Drug Treatment.** All cells were ob-
tained from American Type Culture Collection. Cells were
maintained in RPMI 1640 supplemented with 10% FCS, 2 mM
glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and
25 mM HEPES (Gemini, Woodland, CA). Depo-Provera
was purchased from Pharmacia & Upjohn and was diluted in 28.9
mg/ml polyethylene glycol (PEG, MW 3350), 0.15 M NaCl. All
other drugs were purchased from SIGMA (St. Louis, MO). 100
mM MPA stock solutions were prepared in 80% ethanol, 20% 
chloroform.

For Western blot experiments, 8 × 10^4 cells were plated in
6-well plates 24 h before treatment. Cells were incubated in
serum-free medium supplemented with 0.5% CSS (Sigma) and
treated with either dexamethasone, MPA, Depo-Provera, pro-
gesterone, prolactin, indomycin, NS398, RU-486, or vehicle
(ethanol or polyethylene glycol) at the indicated concentrations.
Cells were harvested after 24–96 h culture as indicated. Whole
cell lysates were prepared as described previously (37). Nuclear
extracts were prepared according to a modified Dignam's pro-
cedure as described previously (38). For Northern blot experi-
ments, 6 × 10^6 cells were seeded in 10-cm² plates in complete
culture medium 24 h before treatment. Drug or vehicle was
added, and cells were cultured for an additional 24–96 h. Cell
monolayers were rinsed in PBS, lysed in TriZol (Invitrogen,
Carlsbad, CA) and phenol/chloroform extracted. An equal vol-
ume of 70% ethanol was added to the extracted aqueous phase,
the solution was applied to an RNAeasy midi column (Qiagen,
Valencia, CA), which was washed and eluted in diethyl pyro-
carbonate-H2O.

**Western Blotting.** Ten μg of cell or nuclear extracts
were electrophoresed on 8–16% Tris Glycine gels (Invitrogen).
After electrottransfer, polyvinylidene difluoride membranes
were divided based on molecular weight markers and incubated
with anti-Nm23-H1 antibody 11 (1:500; Lab Vision/Neomarker,
Austin, TX). Detection of

**Northern Blotting.** Ten μg of total RNA were used for
Northern blotting with Ambion Northern Max kit (Ambion,
Austin, TX). Detection of nm23-H1 transcripts was performed
using a full-length nm23-H1 double-stranded DNA probe, la-
beled with ³²P]dATP (Amersham) using the Prime It Kit
(Stratagene, La Jolla, CA). Filters were exposed to autoradiog-
raphy film using intensifying screens at −70°C for typically
24–72 h.

**Promoter Mutagenesis.** The pBA and pBT plasmids
containing nm23-H1 promoter fragments, as well as the CTF/
NF1 mutant of pBT were described previously (38). To generate
the pBTmGRE plasmid, which contained mutated GREs, the
Quickchange site-directed mutagenesis kit (Stratagene) was
used on the pBT construct. The following primers showing
the mutated sites in bold were used: mGRE-5, 5’-CTGCAG-
AAGAGTCTTTAGATGACTGCC (wild type, CTGCA-
GAAAGTGTTCTCATAAGATGACTGCC); and mGRE-3, GT-
GGGCGAGCGGACTTTAGCACGAAATGGGGCCT (wild type,
GTGGGCGAGCGGACTTTAGCACGAAATGGGGCCT). The gen-
erated mutants were sequence verified.

**Transfections and Transcriptional Analysis.** Cells
(8 × 10^4) were seeded in 6-well plates 24 h before transfection
with 1 μg of pBT construct or variants thereof and 0.3-μg pRL-TK (encoding Renilla luciferase; Promega, Madison, WI) using the Effectene reagent (Qiagen). After 24 h, the transfected cells were treated with the indicated doses of MPA, dexamethasone, or vehicle in serum-free medium supplemented with 0.5% CSS. Cells were harvested at the times indicated and assayed for dual-luciferase activity using the Dual Luciferase Assay System according to the manufacturer’s instructions (Promega). Results are representative of two to three independent experiments in triplicate. Transient transfection of the pBA plasmid and cell culture was performed as described above. CAT and β-galactosidase assays were performed as described previously (38). Data represent triplicate determinations of duplicate experiments.

Soft Agar Colonization Assays. Cells (2 × 10³) were plated in 0.5 ml of culture medium containing 0.3% (w/v) top agar in 24-well plates as described previously (7). At the time of seeding, the culture was supplemented with the indicated doses of MPA or vehicle. After 14 days of culture, colonies (>50 cells) were counted. Results are representative of three independent experiments in triplicate.

Proliferation Assays. Cells were seeded in 96-well plates at 5000 cells/well and allowed to grow for 24 h. The medium was changed and supplemented with the indicated drugs or vehicle, and the proliferation assay was performed 72–96 h later using the cell titer 96 nonradioactive cell proliferation assay from Promega, according to the manufacturer’s instructions. Data are representative of three independent experiments in quintuplicate.

Statistical Analysis. Differences between paired samples were calculated using a two-tailed student’s t test, using the Prism 3.0 software or using ANOVA. Densitometry was performed using the α Innotech ChemiImager 4400, normalizing Nm23-H1 bands to the tubulin control.

RESULTS

Dexamethasone Elevation of Nm23-H1 Expression in Metastatic Breast Carcinoma Cell Lines in Vitro. In the experiment shown in Fig. 1, four human breast carcinoma cell lines were incubated for 4 days in CSS-containing culture medium supplemented with varying concentrations of the glucocorticoid dexamethasone. Nm23-H1 expression was determined on Western blots using an antitubulin hybridization as a loading control. The MDA-MB-231 cell line is metastatic in vivo (14,39). A 2-fold elevation of Nm23-H1 expression was observed in response to 100 nm dexamethasone, and an additional elevation to 3-fold was observed at 1–10 μM dexamethasone, with a high-dose inhibition. For metastatic MDA-MB-435 cells (39), the dose response showed 2.6-fold elevation at >10 nm, with inhibition at >1 μM dexamethasone. Similar trends were observed for Nm23-H2. No change in Nm23-H1 or Nm23-H2 expression was observed in the MCF-7 or ZR-75 cell lines in response to dexamethasone treatment. These cell lines are virtually nonmetastatic to the lungs in vivo (40) and express high endogenous Nm23-H1 levels (38).

Additional characterization of glucocorticoid-induced Nm23-H1 expression is shown using MDA-MB-231 cells in Fig. 2. Another glucocorticoid, prednisolone, elevated Nm23-H1 expression in vitro in the 500 nm to 10 μM dose range (Fig. 2A). Nm23-H1 expression was not elevated by incubation with 100–250 nm prolactin, a hormone that contributes to MMTV regulation via a distinct set of transcription factors (Fig. 2B). Dexamethasone possesses both anti-inflammatory and glucocorticoid properties. To determine whether an anti-inflammatory compound could influence Nm23-H1 expression, MDA-MB-231 cells were incubated in FCS-containing culture medium with two Cox inhibitors, indomethacin or NS398 (41). Neither agent altered Nm23-H1 expression as determined by Western blots (Fig. 2C), and similar effects were observed when these agents were added to CSS-containing culture medium supplemented with glucocorticoids (data not shown), in agreement with Natarajan et al. (35). The data are consistent with a glucocorticoid-based mechanism of action. Time course experiments indicated that dexamethasone elevation of Nm23-H1 expression required 4 days of exposure (data not shown).

The high-dose inhibition of the dexamethasone effect (Fig. 1) suggested that pharmacological dexamethasone concentrations may be ineffective at elevating Nm23-H1 expression. Additional support for this conclusion is provided in the experiment shown in Fig. 3 in which dexamethasone failed to elevate...
Nm23-H1 expression in FCS-containing cultures, which contain endogenous levels of corticosteroids. As this finding limited the potential in vivo usage of dexamethasone as a Nm23-H1 elevating agent, we searched for a GR agonist with a different mechanism of action.

**MPA Elevation of Nm23-H1 Expression.** MPA is well known as a PR agonist, but also has been reported to bind GR and the AR. MPA also affects glucocorticoid responses through a posttranscriptional mechanism involving GR, independent of GRE binding (42–44). On the basis of this mechanistic diversity, we asked if MPA elevated Nm23-H1 expression and if it did so with an altered dose response or serum requirement. MDA-MB-231 and MDA-MB-435 breast carcinoma cell lines were determined to be PR—by both Western and reverse transcription-PCR analyses (data not shown). In the experiments shown in Fig. 4 MDA-MB-231 and MDA-MB-435 cells were incubated for 3 days in varying doses of MPA in either CSS- or FCS-containing medium, and the Nm23-H1 expression of the cell lysates determined on Western blots. MPA elevated Nm23-H1 expression in both cell lines under both culture conditions in contrast to dexamethasone (Figs. 1 and 3). Elevation of Nm23-H1 expression was observed at higher MPA concentrations in FCS-containing cultures, ≥3-fold at 10 nM to 10 μM for MDA-MB-435 cells and 3-fold at 1 μM for MDA-MB-231 cells. MPA had no effect on the Nm23-H1 expression of MCF-7 and ZR-75 cells (data not shown). Both MPA and clinical grade Depo-Provera elevated Nm23-H1 expression with comparable dose response curves (data not shown).

**Mechanistic Studies.** Transiently transfected reporter gene constructs were used to determine the transcriptional effects of dexamethasone and MPA on nm23-H1 expression using MDA-MB-231 breast carcinoma cells. In the experiment shown in Fig. 5A, MDA-MB-231 cells were transiently transfected with pBT, a plasmid containing a 443-bp nm23-H1 promoter tethered to Firefly luciferase and cultured 4 days in 0.5% CSS-containing medium. Transfection efficiency was normalized by cotransfection of a Renilla luciferase plasmid. MDA-MB-231 cells cultured in the presence of 10 nM dexamethasone showed an 80% increase in reporter gene expression as compared with vehicle controls or cells cultured with 50 ng/ml prolactin, in agreement with Western blot data (Figs. 1 and 2). Mutation of two GREs in pBT abolished the dexamethasone response (Fig. 5B). In experiments not shown, mutation of the CTF/NF1 half-site in the nm23-H1 promoter also abolished dexamethasone stimulation of reporter gene expression, in agreement with its contributory role in nm23 expression (38) and crucial role in mediating glucocorticoid responses in MMTV (45). Also, cotransfection of a GR plasmid did not alter the effect of dexamethasone on nm23-H1 transcription, indicating that maximum functional GR was present endogenously (data not shown). Similar conclusions were obtained when using MPA over a shorter time course (Fig. 5C). MPA stimulated reporter gene expression by 88% at 48 h of culture, a time point when dexamethasone was inactive. Mutation of the GREs abolished this response. Both dexamethasone and MPA also stimulated reporter gene expression using a different reporter construct containing a longer (2.1 kb) fragment of the nm23-H1 promoter (Fig. 5D). These data are consistent with a transcriptional elevation of nm23-H1 expression by dexamethasone and MPA for cells cultured in CSS-containing medium, with MPA exerting a more rapid effect over time. In contrast, no elevation of nm23-H1 reporter gene expression was observed in FCS-containing cultures using constructs containing either the 2.1- or 443-bp promoter fragments, in response to 1 nM to 1 μM MPA over a period of 1–5 days of culture (data not shown). In agreement with these data, no elevation of nm23-H1 mRNA levels was observed in response to 1 nM to 1 μM MPA treatment of MDA-MB-231 cells cultured in FCS-containing medium on Northern blots (data not shown). These data suggest that dexamethasone and MPA can increase Nm23-H1 expression transcriptionally, but in the presence of endogenous levels of corticosteroids, additional posttranscriptional mechanisms must be operative for MPA.
The time course of MPA elevation of Nm23-H1 expression required 72 h of culture (Fig. 6A). Re-}
pressed the interleukin 2 promoter via a GR-related mechanism in T lymphocytes, without demonstrable stimulation of a GRE reporter construct. In another cytokine model system, MPA decreased uterine smooth muscle cell production of interleukin-1β without affecting the transcriptional activation of its promoter construct (46). Finally, MPA up-regulated p27kip1 expression in human endometrial cells, without an increase in p27 mRNA, similar to data presented herein. Decreased degradation of p27 protein in response to MPA was observed (47). The glucocorticoid literature identifies additional potential points of posttranscriptional regulation that could be applicable, including the modulation of interacting cyclic AMP, nuclear factor-xB, Grb2, and G-protein-mediated pathways (48–51), and intracellular redistribution of signaling components (52). Our data indicate that MPA elevation of breast carcinoma Nm23-H1 expression was GR dependent and PR-independent because the cell lines used herein were PR− and GR+ by Northern and Western blot analyses.

MPA inhibited the soft agar colonization of both metastatic breast carcinoma cell lines by ~50%. Although soft agar colonization may be representative of several aspects of tumor progression in vivo, we suggest that it may model the colonization of single metastatic cells in a distant organ, independent of primary tumor cell:cell, cell:extracellular matrix, and cell:local cytokine interactions (2). We previously reported that the colonization of MDA-MB-435 breast carcinoma cells was stimulated by addition of TGF-β and that nm23-H1 transfectants continued to colonize poorly under these conditions (7). Our recent experiments showed variability in TGF-β stimulation of colonization: of 10 experiments conducted using 1–10 ng/ml TGF-β, 2 resulted in >2-fold stimulation of colonization, 3 resulted in 40–50% stimulation of colonization, and the rest
We plan to study the effects of MPA GR. Effects on both the host and tumor cells have been reported. MPA effects may be cell type specific and within a cell type angiogenic factors (58, 59), and apoptosis (55, 60). Clearly, to affect insulin-like growth factor binding protein levels (57), mones or chemotherapeutic agents (53–56). MPA is also known to stimulate growth factor production via the PR in breast cancer trials, no maximum-tolerated dose or optimal schedule was agreed upon (reviewed in Ref. 70). As our translational hypothesis concerning elevation of Nm23-H1 expression would entail the chronic administration of MPA or a similar agent, we have examined two recently reported clinical trials using longer term dosing. A randomized trial, including 270 node-positive patients with median 13 years of follow-up, was reported (71). Node-positive patients were given cyclophosphamide, methotrexate, 5-fluorouracil chemotherapy and randomized to 6 months of placebo or MPA, consisting of a 1-month induction schedule followed by a maintenance dose. No difference was noted in the overall clinical course of the node-positive patients. However, when patients were dichotomized by age, older patients (≥50 years) showed a significantly prolonged relapse-free (P = 0.01) survival on the MPA arm; the molecular specificity, have subsequently been found to exert additional activities, which may be beneficial or adverse (61, 62). Second generation, more specific metastasis suppressor elevating agents would represent a potential advance.

In mouse breast cancer model systems, two distinct effects of MPA have been reported. In vivo, MPA inhibited rat 7,12-dimethylbenz(a)anthracene mammary tumor formation (63) and suppressed the proliferation and differentiation of MCF-7 tumor cells (64). In contrast, MPA-induced murine mammary tumors have been reported previously (65, 66). MPA has been reported to stimulate growth factor production via the PR in breast explants, suggesting that the stimulatory effect may be PR driven (67). In humans, MPA is used at low doses in the oral contraceptive Depo-Provera, with a long-term record of low toxicity (68). On the basis of the reports of stimulatory effects of MPA on cancer in rodents, an analysis of pooled case-control studies of Depo-Provera in humans concluded that the relative risk for breast cancer among women was insignificant (relative risk, 1.1; 95% confidence interval, 0.97–1.4; Ref. 69). At higher doses, MPA has been tested in clinical trials against metastatic breast and uterine cancers. Although clinical responses were reported in breast cancer trials, no maximum-tolerated dose or optimal schedule was agreed upon (reviewed in Ref. 70). As our translational hypothesis concerning elevation of Nm23-H1 expression would entail the chronic administration of MPA or a similar agent, we have examined two recently reported clinical trials using longer term dosing. A randomized trial, including 270 node-positive patients with median 13 years of follow-up, was reported (71). Node-positive patients were given cyclophosphamide, methotrexate, 5-fluorouracil chemotherapy and randomized to 6 months of placebo or MPA, consisting of a 1-month induction schedule followed by a maintenance dose. No difference was noted in the overall clinical course of the node-positive patients. However, when patients were dichotomized by age, older patients (≥50 years) showed a significantly prolonged relapse-free (P = 0.01) survival on the MPA arm; the
reverse was true of younger patients. A similar conclusion was reached by a second group with 12 years of follow-up (72). A total of 409 patients was treated with adjuvant cyclophosphamide, doxorubicin, 5-fluorouracil chemotherapy and randomized to a 6-month course of MPA, also given as an induction and maintenance schedule. No difference in survival was noted for the group as a whole, but subset analysis again noted better metastasis-free (P = 0.01) and overall (P = 0.02) survival for older patients (≥60 years). These trends suggest a beneficial effect of long-term MPA dosing in a postmenopausal patients. It may be of interest to determine whether patients with low Nm23-H1, GR+, PR—tumors responded better.

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REFERENCES


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