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13. ABSTRACT ***(Maximum 200 Words)***

Environmental pollutants such as polycyclic aromatic hydrocarbons (PAH) are believed to contribute to the recent increase in breast cancer incidence and mortality. Yet, the molecular mechanism is poorly understood. In this study, we use a carcinogen-induced breast cancer animal model in which the female Sprague-Dawley (S-D) rats develop mammary tumors after a single intragastric dose of treatment of 7,12-dimethylbenz(α)anthracene (DMBA), a member of the PAH family. Estrogen is indispensable for the DMBA-mediated mammary tumorigenesis. We hypothesize that DMBA and estrogen receptor (ER) cooperate in activation of protooncogene *Mdm2* which is important for DMBA-rat mammary tumorigenesis. We have confirmed that MDM2 is indeed overproduced in DMBA-mammary tumors, likely via a post-transcriptional mechanism(s). Analysis using the Rat cDNA Expression Array indicates an up-regulation of p450, as expected, as well as the down regulation of IGF1, IGF1R, c-Myc and JNK in the DMBA-treated mammary glands at an early stage of the DMBA treatment. We have also found a clear correlation between MDM2 expression and the status of the aromatic hydrocarbon receptor (AhR) and ER in a number of human breast cancer cells. MDM2 expression in MCF-7 cells is activated in a DMBA dose- and time-dependent manner. We show that at least two cellular proteins can specifically interact with an AhR site in MDM2 5' UTR. We conclude that overproduction of MDM2 may play a pathological role in carcinogen-induced mammary tumorigenesis and that MDM2 is upregulated by AhR and ER independent of p53 action.

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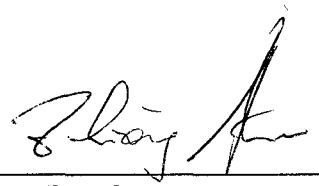

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INTRODUCTION

This project aims at elucidating the mechanism(s) by which the environmental carcinogen, 7,12-dimethylbenz(α)anthracene (DMBA), selectively induces mammary gland tumors in female Sprague-Dawley (S-D) rats. In particular, we plan to investigate the effects of DMBA on the expression and functions of certain key tumor suppressor genes and cell cycle regulators. These genes include murine homologue of the human breast cancer susceptibility gene 1 (Brca1), murine double minute (Mdm2) and p53. In addition, we plan to study the mechanism of estrogen-mediated BRCA1 and MDM2 activation. In the project, we proposed three technical objectives: (1) Analysis of the temporal effects of DMBA on the expression of Brca1, Mdm2 and p53; (2) Study of the effects of DMBA on the integrity of Brca1, p53 and Mdm2; and (3) The role of CBP/p300 in the ER-mediated regulation of Brca1 and Mdm2. Information obtained from this study may provide insights how environmental carcinogen promotes human breast cancer.

In the past year, we have made substantial progress on all the Technical Objectives. These progress include that (1) we have began to analyze the effect of DMBA-treatment on gene expression in rat mammary tissue using cDNA Expression Array technology; (2) we have confirmed that MDM2 over production in DMBA-rat tumors by immuno-staining; (3) we have recaptured the DMBA effects on MDM2 expression using the human MCF-7 breast cancer cells (4) based on the newly identified putative aromatic hydrocarbon receptor (AhR)-binding site in the 5' untranslated region of MDM2 gene, we have performed EMSA to demonstrate the functionality of the AhR site and (5) we have shown a positive correlation between AhR and MDM2.

The work-in- progress provides a solid basis for accomplishing and extending the studies proposed in this projects funded by the Breast Cancer Research Program of Department of Defense.

BODY

a. Experimental Methods

a.1. Animal treatment:

The animal treatment with vehicle or DMBA has been described in my last annual report based on the protocol developed by Dr. Rogers (Lee et al., 1987; Lee et al., 1986; Rogers, 1989). Briefly, a group of female S-D rats with equal average weight will be randomly chosen to give a 25 mg/kg dose of DMBA in 0.2 ml sesame oil by gastric gavage at age of 56 days, which resulted an average latency of 8-12 weeks and a total tumor incidence of 65-85% at 12-20 weeks with 3 to 5 tumors per tumor-bearing rat (Rogers and Conner, 1990). The other group was given only the oil vehicle. The rats were sacrifice in a group of 5 at 6 hours, 24 hours, or 1, 9 weeks after DMBA or oil exposure. The right and left second, third and fourth mammary glands were rapidly excised and frozen as described (Lee et al., 1987). All the tumors were weighed and grossly examined. The segments of tumors and normal mammary glands were frozen for histological, biochemical, immunohistochemical studies. The animal treatment was performed by Drs. Adrianne Rogers and Gail Sonenshein, our collaborators for this project.

a.2. Isolation of RNA, DNA and proteins from mammary tumors and normal glands

Frozen rat mammary tissue samples were grounded into powder in liquid N2, and processed for isolation of total RNA, DNA and protein using Trizol reagent according to the manufacture's instructions (Gibco). 200 to 500 mg of tissue was homogenized (1 ml of Trizol per 100 mg of tissue dry powder) using a Wheaton 15 ml Dounce Tissue Grinder. After brief centrifugation to remove the insoluble material and separate the fat layer, 0.2 ml of chloroform per 1 ml of Trizol was added to the cleared homogenate. After vigorous shaking and centrifugation at 12,000 x g for 15 minutes at 4°C, total RNA in the aqueous phase was precipitated by isopropyl alcohol. DNA from the organic phase and the interphase was precipitated with ethanol. The remaining supernatant was precipitated with isopropyl alcohol for isolation of proteins.

The resulting RNA pellet was resuspended in DEPC-treated water and stored at -80°C. The DNA pellet was first resuspended in 8 mM NaOH, then adjusted to pH 7.6 using 1M HEPES buffer and stored

at -20°C. The protein pellet was sonicated in a buffer of 1% SDS, 250 mM NaCl, 50 mM Tris and stored at -20°C.

The yield is 2 - 5 μg, 0.5 - 1.5 μg and 3 - 6 μg per mg tissue powder for RNA, DNA and protein, respectively. The reproducible high quality of each preparation has been examined and confirmed by Northern, RT-PCR, Southern and Western blot analyses, respectively.

a.3. Western blot analyses

Thirty to fifty μg of total protein was loaded per lane, separated on SDS-PAGE and transferred on Immobilon-P membrane. The membrane was blocked in 5% dry milk and incubated with primary antibody (purchased from Santa Cruz) specific for p53 (DO1), RB (IF8), p27 (C-19) and p21 (purchased from Pharmingen). The specific proteins were visualized using an ECL procedure (Amersham).

a.4. DNA Atlas

The CLONTECH's Atlas Rat cDNA expression Arrays were used according to the manufactures' instruction. Briefly, one μg of poly-A^+ RNA isolated from rat mammary tissue samples was used in the cDNA synthesis Primer Mix in the presence of MMLV reverse transcriptase and [a-^{33}P]dATP. 1 x 10^6 cpm of ^{33}P-labeled, column-purified cDNA probes was used in probing the Rat cDNA Arrays. The autoradiography was initially scanned visually.

a.5. DMBA-treatment

Human breast cancer cell lines MCF7 and Hs578T were grown to 60-70% confluence in 6-well plates using DMEM (4.5 g/L glucose, in the absence of phenol red) containing 10% of charcoal/dextran treated FBS (Gibco). DMBA was added at the concentration as specified. Cell lysates were collected after 12 - 16 hours DMBA- or vehicle-treatment.

a.6. EMSA

Nuclear extracts from MCF-7 or Hs578T cells were prepared using the procedure as described before. Five μg extract was used per reaction in the presence of ^{32}P-labled double-stranded DNA oligo probes. EMSA was performed as described.

b. Results

b.1.Alternation of Gene Expression at the early stage of DMBA treatment.

After we observed the upregulation of cyclin D1 and MDM2 in the DMBA-rat mammary tumors using RT-PCR, and Western analysis (1998 DOD-annual report), we have confirmed the overproduction of MDM2 in the DMBA-tumors by immunohistochemical staining using the MDM2-specific monoclonal antibody 2A10 (data not shown).

Since tumorigenesis is multiple progress including initiation, promotion and progression, it is of great interest to examine the expression of genes at the various stages of the tumorigenesis. We therefore determined to test the gene expression of DMBA- or vehicle-treated mammary tissue samples at 6 hours of treatment by Rat cDNA expression Array. As shown in Figure 1, certain genes are upregulated by DMBA treatment, such as p450 (Figure 1, mark 1), as expected. Interestingly, ERK1 gene is also upregulated (Figure 1, mark 2). Some genes are down regulated including IGF1 (Figure 1, mark 3), IGF1Rα (Figure 1, mark 4), c-Myc (Figure 1, mark 5), H-Ras (Figure 1, mark 6), CDK5 (Figure 1, mark 7), bFGFR (Figure 1, mark 8) and JNK (Figure 1, mark 9). We are at present in the process of repeating the same experiments, quantitating the signals on the cDNA arrays and analyzing samples obtained during the time course of DMBA-induced mammary tumorigenesis. We are unable to assess the expression of MDM2 since the cDNA array does not contain the rat MDM2 gene.

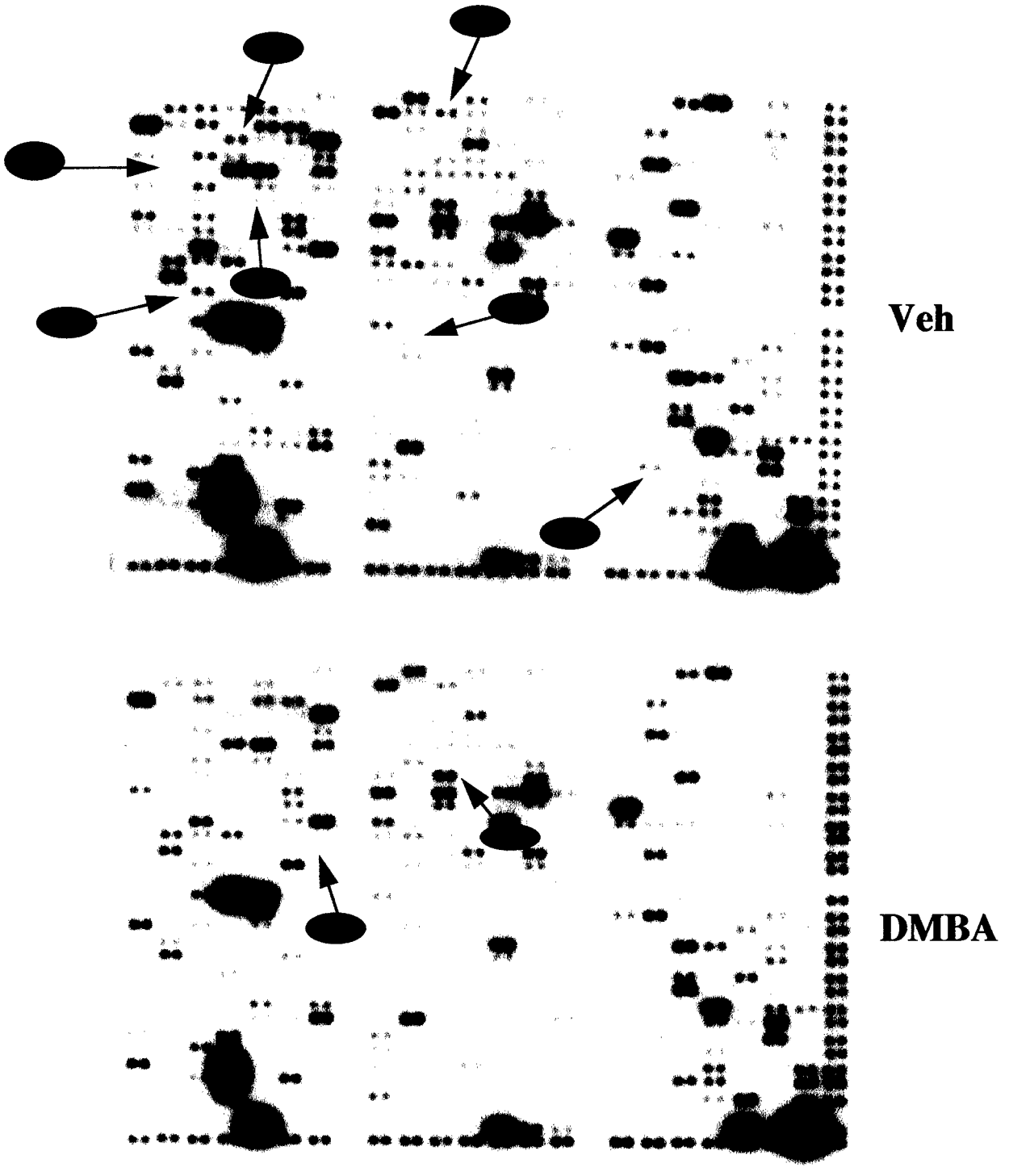

Fig. 1 Atlas of Gene Expression in DMBA-treated Rat Mammary Tissues at 6 Hours.

b.2. DMBA activates MDM2 expression in MCF-7 cells in does and time-dependent manner.

We have identified a putative AhR/ARNT site in the 5' UTR of MDM2 gene (Figure 2. Also, see 1998 DOD annual report). It is known that the action of DMBA is to bind its receptor AhR/ARNT to translocate into nucleus which then bind to AhR/ARNT sites to transactivate genes. Given the observed MDM2 overexpression in the DMBA-tumors, it is of great interest to explore whether DMBA can directly activate MDM2 gene expression in human breast cancer cells. Therefore, we treated the breast cancer cells MCF7 (p53+, ER+) or Hs578T (p53-, ER-) with an increasing amount of DMBA (0, 1, 10, 50 μM) for 12 hours, and the immunoblotting analyses on MDM2, p53, p21, p27 and RB were performed using the whole cell lysates. Clearly, the steady-state levels of MDM2 protein in MCF7 cells, but not in Hs578T cells, was significantly higher in a DMBA dose-dependent manner (Figure 3A). In contrast, neither RB nor p21, nor p27 protein levels were significantly altered. There is no significant change in p53 protein level at this condition. When tested the time course effect of DMAB (10 μM) on the expression of MDM2, it is apparent that the upregulation of MDM2 is evident starting at 12 hours after DMBA treatment (Figure 3B). Both p53 and p21 appear to be up regulated albeit at much less extent (Figure 3B).

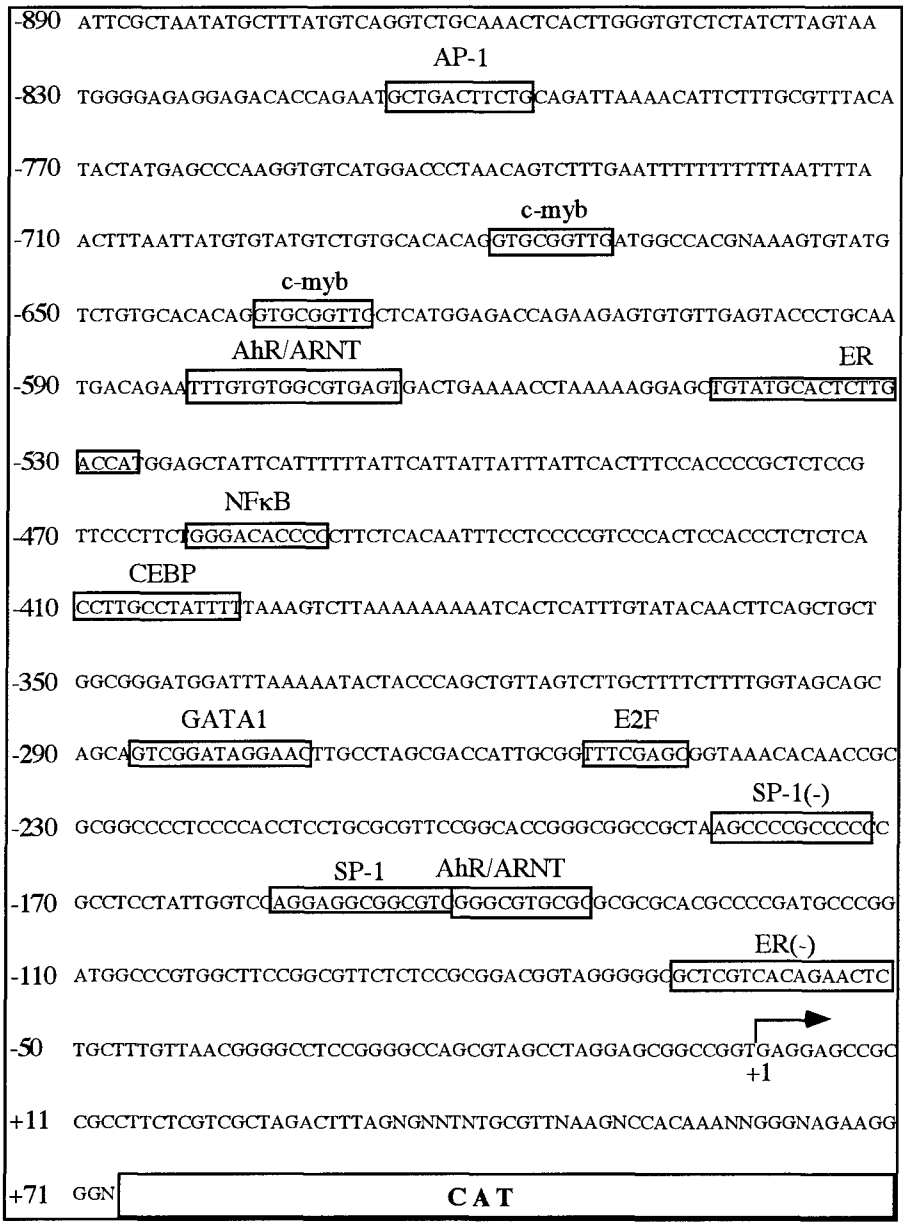

Figure 2. Some putative regulatory elements in the 5' UTR of human MDM2 gene. The putative regulatory elements match 100% with the core sequences and 80% or above the matrix consensus sequence (Quandt et al., 1995)

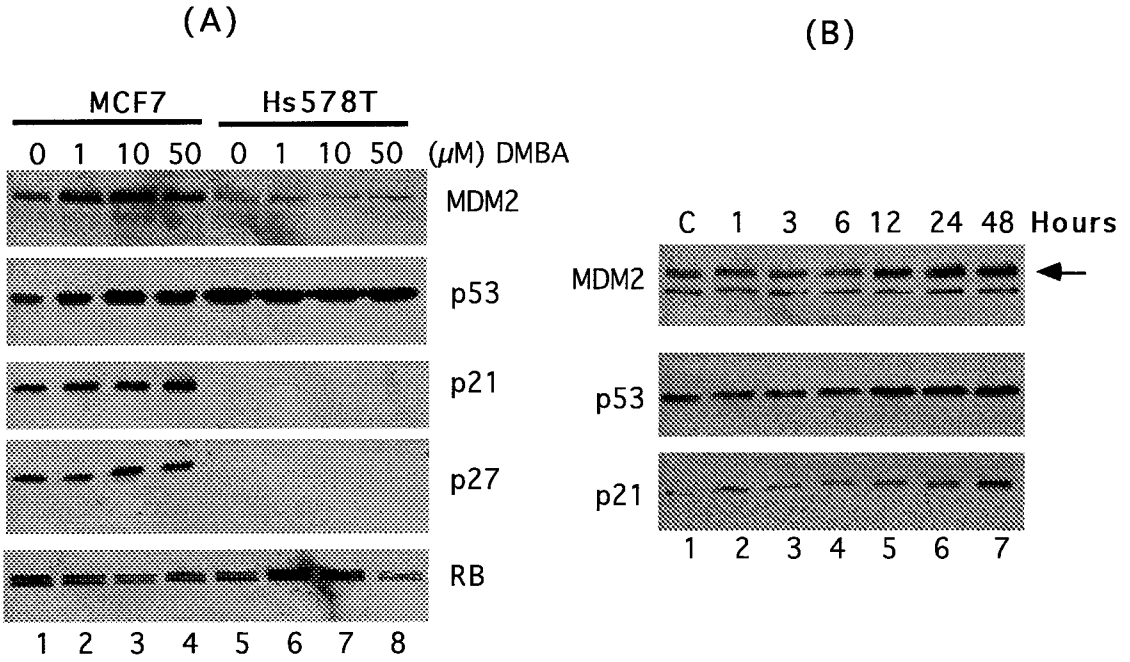

Figure 3. DMBA activates MDM2 expression in MCF7 cells in a dose- and time-dependent manner. Thirty to fifty μg of total protein was loaded per lane. The membrane was with probed with antibody (from Santa Cruz) specific for p53 (DO1), RB (IF8), p27 (C-19) and p21 (Pharmingen). Specific proteins were visualized using an ECL procedure (Amersham).

b.3. MDM2 expression correlates with the expression of AhR.

Since MDM2 gene contains a AhR/ARNT binding site, which implies that AhR might directly regulate MDM2 regulation in response to DNA damage, we therefore examined the relationship between the expression of MDM2 gene and AhR gene. Western blot analysis was performed to accesses MDM2 expression in three human myeloma cell lines: Sultan, IMP and U266. Both Sultan and IMP cells express high levels of AhR while U266 cells express little if any AhR (Dr. D. Sherr, personal communication, and data not shown). As shown in Figure 4, treatment of DMBA, but not the vehicle (acetone), on both Sultan and IM9 cells led to a substantial increase in the expression of MDM2. Treatment of ANF, which antagonizes DMBA significantly, reduced the effect of DMBA on MDM2 expression. However, MDM2 expression in the AhR negative U266 cells was barely detectable and DMBA treatment led no significant change.

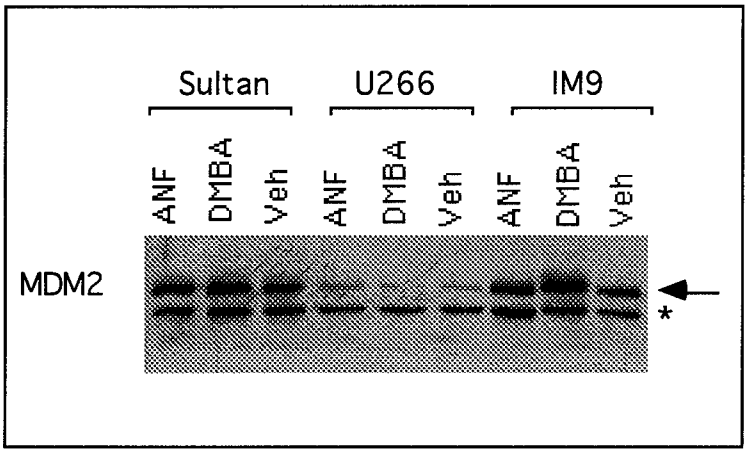

Figure 4. Expression of MDM2 correlates with AhR. 50 μg total protein was loaded per lane. Western blot analysis was performed using MDM2-specific antibody (2A10). MDM2 protein is indicated by an arrow. A non-specific protein is also indicated by an asterisk.

b.4. Specific cellular proteins bind to the AhR/ARNT site located in the 5' UTR MDM2 gene (-581 to –566).

Next, we asked where the AhR/ARNT site is functional in interaction with specific cellular proteins. We used the double stranded DNA oligo (30 mers, designated as P1 probe) which bears identical sequences to (–588/-559) of MDM2 gene containing the AhR/ARNT site (-581/-566) (Figure 1). P1m is mutant oligo which contains the scrambled sequence on the AhR/ARNT site but otherwise identical to P1. EMSA was performed using the ^{32}P-labelled probe (P1) and the nuclear extracts from either MCF-7 cells or Hs578T cells. As shown in Figure 5, at least two specific protein-DNA complexes from both MCF-7 and Hs578T cells were detected when P1 was used as probe. Cold competition assay clearly indicated the specificity of these two protein-DNA complexes (indicated by arrows), as evidenced by the competition of the cold wild-type P1 (Figure 5, lane 4), but not by the cold mutant oligo (P1m) (Figure 5, lane 5). DMBA-treatment of MCF-7 cells appeared to lead an increase of at least one specific protein-DNA complex (Figure 5, lane 6). Whether these protein-DNA complexes contain AhR/ARNT is at present under investigation. Since MDM2 contain another putative AhR/ARNT site (-141/-132) and a putative ER site (-543/--525) (Figure 1), we examined whether these two sites are functional in interaction with cellular proteins. A double-stranded DNA oligo (-165/-136, designated as P2 oligo) covering the AhR/ARNT site (-141/-132) was used in EMSA. Similarly, P3 oligo (30 mers, -548/-519) contains the putative ER site was also tested in the EMSA. Our data indicated clearly that there are no detectable protein-DNA complexes using either P2 (Figure 5, pane B, lane 2) or P3 (Figure 5, panel B, lane 3) oligo as probe.

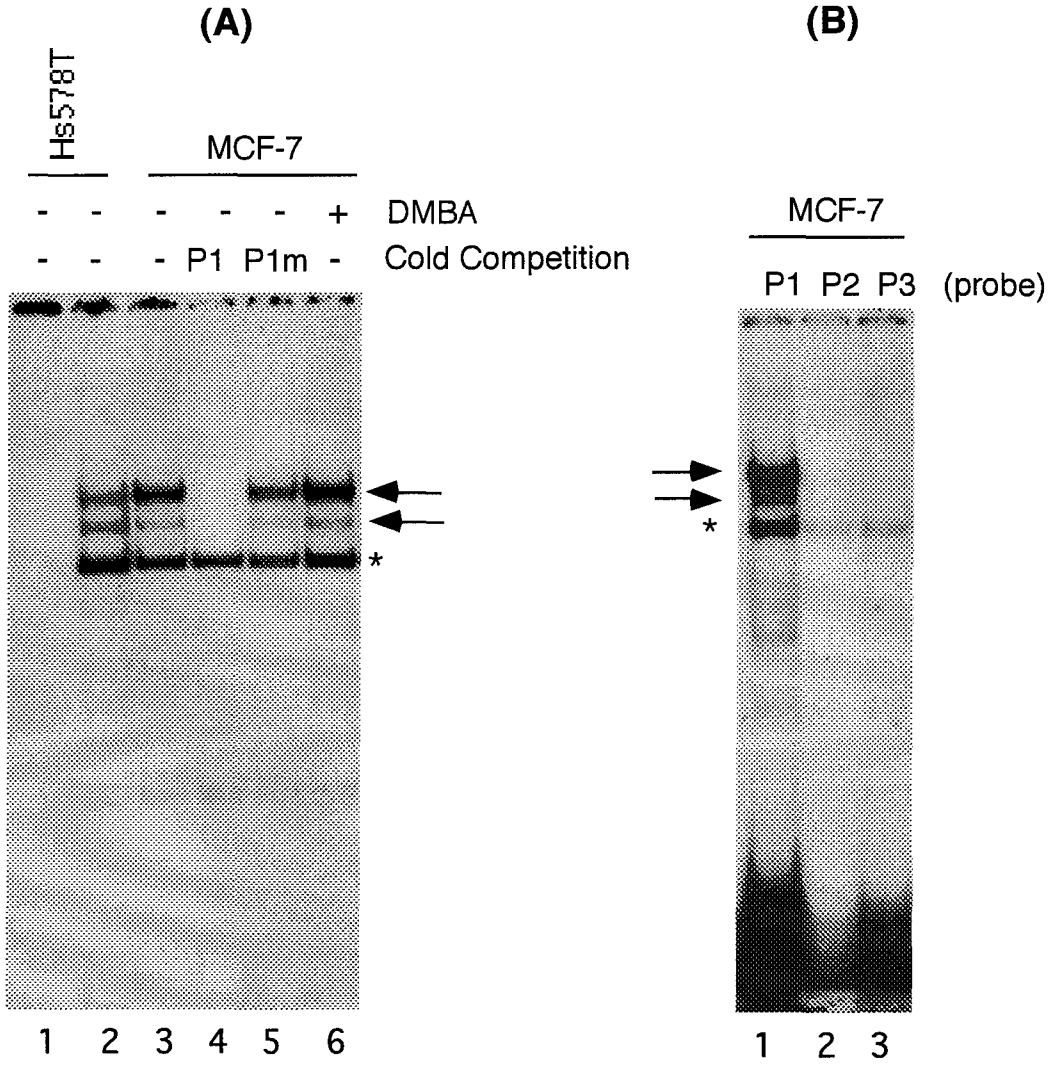

Figure 5. **The AhR/ARNT site (-581/-566) on the MDM2 5' UTR binds to specific cellular proteins**. Specific protein-DNA complexes are indicated by arrows whereas a non-specific protein is indicated by an asterisk.

c. Discussion

MDM2 is overexpressed in DMBA-induced tumors. Our data indicate that MDM2 is clearly over produced in mammary tumors and this overproduction is likely at the post-transcriptional level. It has been reported that MDM2 expression may be translationally up-regulated (Capoulade et al., 1998; Landers et al., 1997; Landers et al., 1994). Recently, it has been reported that bFGF can increase MDM2 protein levels independent of p53 (Shaulian et al., 1997). Importantly, overproduction of MDM2 protein, in the absence of MDM2 gene amplification, has been documented in some human tumors (Bueso-Ramos et al., 1993; Kawamata et al., 1996; Lianes et al., 1994), implying that MDM2 overproduction is not a passive indicator of genomic instability. In contrast, it is likely a causative factor contributing to human tumorigenesis.

Through cDNA expression array, we have detected the alternation of gene expression at the early stage (6 hours) during the DMBA treatment. As expected, the p450 gene is upregulated. P450 is the well characterized gene activated by AhR (Gasiewicz and Rucci, 1991; Hoffman et al., 1991; Nebert and Gonzalez, 1987). Interestingly, ERK1 gene is also upregulated, the biological consequence(s) of the upregulation is not clear. Most notably is the down regulation of certain cell proliferation-related genes, such as c-Myc, H-ras, cdk5 and IGF1. The dada is consistent with the observation that DMBA treatment led to depression of DNA synthesis at the early stage (first day) of DMBA treatment (Lee et al., 1986; Rogers and Conner, 1990). We are currently examining the gene expression profile of time course samples from the DMBA-induced mammary tumorigenesis (24 hours, 1, 3, 9 weeks as well as the mammary tumors) using the rat cDNA arrays. Since the rat MDM2 gene is not included on the cDNA array, we are taking an alternative path, that is, to analyze the MDM2 expression by conventional Northern and Western blot analyses.

Is MDM2 overproduction a p53-mediated phenomenon? Our data does not exclude either one possibility. DMBA activates MDM2 expression in the p53 + MCF-7 cells, but not in the p53- Hs578T cells. In the time course experiments, it seems that p53 is also upregulated which may count for the upregulation of MDM2. However, a set of our data suggest that MDM2 may be upregulated by DMBA-AhR pathway independent of p53. First, MDM2 expression correlates well with the status of AhR expression (Figure 4). Second, there is a functional AhR/ARNT binding site in interaction with cellular proteins, presumably including the AhR and/or ARNT proteins. Third, our preliminary data suggest that ectopic expression of AhR in U266 cells leads to the upregulation of MDM2 (data not shown). All these dada are consistent with the notion that MDM2 is directly activated by DMBA via a p53-independent pathway. Yet, it is also possible that the increased levels of MDM2 proteins in DMBA-tumors is due to an abnormal protein degradation machinery involved in MDM2 protein stability. Whatever the case, it is important that DMBA-mediated mammary tumorigenesis may be, in part, via the activation of MDM2.

In the coming year, we will continue to examine the temporal effects of DMBA on the expression of MDM2, p53, Brca1 among others and will examine whether CBP/p300 plays a role in ER-mediated regulation of MDM2 and Brca1.

KEY RESEARCH ACCOMPLISHMENS

Our work-in-progress has the following major accomplishments:

(1) We have confirmed that MDM2 overproduction is associated with the environmental carcinogen-induced rat mammary tumorigenesis.

(2) We have obtained the valuable mammary tissue samples during the time course of DMBA-induced mammary tumorigenesis, which will be used in examining gene expressions at the genome level.

(3) We have shown that DMBA can activate MDM2 in human breast cancer cells in a dose- and time-dependent manner.

(4) Our data indicate that DMBA may directly activate MDM2 expression trough AhR/ARNT mediated gene activation. Thus, we have identified an alternative pathway that activates MDM2

REPORTABLE OUTCOMES

Presentations:

Z.-X. J. Xiao, "MDM2 and Breast Cancer", invited seminar, Section of Oncology/Hematology, Department of Medicine, Boston University School of Medicine, December, 9, 1999.

Z.-X. J. Xiao, "MDM2, p53 and Breast Cancer", invited seminar, Department of Animal Sciences, University of Massachusetts at Amherst, March, 24, 1999.

Z.-X. J. Xiao, "Environmental carcinogen-induced breast cancer", invited seminar, Program in Research on Women's Program, Boston University School of Medicine, January 5, 1999

Funding applied based on work supported by this award:

Part of the preliminary data generate from this work was used in my NIH R01 application (RO1 CA79804-01A1) entitled "Function of MDM2-RB and its Role in Breast Cancer", which is funded by NCI through 07/01/19999 to 04/40/2004.

CONCLUSION

Environmental pollutants such as polycyclic aromatic hydrocarbons (PAH) are believed to contribute to the recent increase in breast cancer incidence and mortality. Yet, the molecular mechanism of the breast tumorigenesis is poorly understood. In this study, we have employed a carcinogen-induced breast cancer animal model in which the female Sprague-Dawley (S-D) rats develop mammary tumors within 12-20 weeks of a single intragastric dose of treatment of 7,12-dimethylbenz(α)anthracene (DMBA), a member of the PAH family. Estrogen is indispensable for the DMBA-mediated mammary tumorigenesis. To test the hypothesis that DMBA and estrogen receptor (ER) cooperate in activation of protooncogene *Mdm2* in the DMBA-rat model, we have analyzed RNA and protein levels from mammary glands collected from DMBA- or vehicle-treated control animals at 6 or 24 hours, and 1, 3, 9 or 18 weeks following the treatment. Our data indicate that cyclin D1, MDM2 and p27 are overproduced in the mammary tumors whereas there is no significant change in the expression of p16, p53 and RB. Cyclin D1 overproduction is due to an increased steady-state levels of mRNA whereas a post-transcriptional mechanism(s) is responsible for overproduction. MDM2 and p27. DNA Atlas Analysis shows an up-regulation of p450 and ERK1 but down regulation of IGF1, IGF1R, c-Myc and JNK in the DMBA-treated mammary glands at an early stage of the treatment. When a number of human cancer cell lines are examined, the MDM2 expression is found to closely correlate with the expression of the aromatic hydrocarbon receptor (AhR) and ER. In addition, MDM2 expression in MCF-7 cells is activated in a DMBA dose- and time-dependent manner. DNA sequence analysis of MDM2 5' promoter region reveals several putative ER and AhR/ARNT binding sites. At least two cellular proteins are shown to specifically interact with an AhR site in the MDM2 promoter as indicated by EMSA. Co-transfection of ER in the presence of E2 leads to an activation of a MDM2-CAT reporter. We conclude that overproduction of MDM2 may play a pathological role in carcinogen-induced mammary tumorigenesis and that MDM2 is upregulated by AhR and ER independent of p53 action.

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MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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