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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-mediated rat mammary tumorigenesis involves in the activation of protooncogene *Mdm2* which in turn negatively regulates the tumor suppressor protein p53. We have confirmed that MDM2 is indeed overproduced in DMBA-mammary tumors by Western blot analysis and immunohistochemical staining. 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. Moreover, we have evidence that IGF-1 protects DNA damage-induced cell death by upregulation of p21 and MDM2. We conclude that overproduction of MDM2 may play a pathological role in carcinogen-induced mammary tumorigenesis, that MDM2 is upregulated by AhR and ER independent of p53 action.

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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 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.

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 immunostaining; (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.

Data presented in this final report has laid a solid foundation for further study of the project 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. RT-PCR

For the reverse transcription, 2 μg of oligo-dT (Gibco) and 4 μg of total RNA in a total volume of 22 μl DEPC-treated water were used. After heating at 70°C for 10 minute, 8 μl of 5X RT buffer, 4 μl of 0.1 M DTT, 4 μl of dNTPS (5 mM) and 2 μl of SuperScript reverse transcriptase (Gibco) were added. The reaction was proceeded for 1 hours at 37 °C and terminated by heating at 70°C for 5 minutes.

Two μl of the RT-products was used for PCR reaction. The oligonucleotide primers for murine cyclin D1 are D103 (5'CAGACGGCCGCGCCATGGAA3') and D105 (5' AGGAAGTTGTTGGGGCTGCC3') (Sicinski et al., 1995). The predicted size of the PCR product is 681 bp. As for murine MDM2, the primers used in the PCR are M109 (5'ATGTGCAATACCAACATCTCTGTGTC3') and M110 (5'GCTGACTTACAGCCACTAAATTTC3') (Jones et al., 1995). The predicted PCR product is 314 bp. The condition for PCR reaction was 40 sec at 94°C, 1 min at 60°C and 1 min at 72 °C for 30 cycles followed by final cycle for 7 minutes at 72°C. The PCR products were run on a agarose gel and visualized by ethidium staining.

a.4. 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.5. Immunohistochemical analysis

All staining was performed using the Vectastain ABC kit (Vector) according to the manufacturer's specification. Briefly, formalin-fixed, paraffin embedded tissues were sectioned at 5 μM, mounted, and deparaffinized. Sections were incubated for 30 minutes in a solution of 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Antigen retrival was then performed by boiling sections in a 1X Antigen Unmasking Solution (Vector) for 10 minutes. After cooling at room temperature for 30 minutes, sections were washed for 10 minutes in PBS and blocked for 20 minutes with normal horse serum in PBS. MDM2 monoclonal antibody 2A10 was diluted 1:200, added to sections, and incubated overnight in a humid chamber a 4 C. After a 10 minute PBS wash, diluted biotinylated secondary antibody (horse anti-mouse) was added for 30 minutes at room temperature. Following a 10 minute wash in PBS, sections were incubated in Vectastain ABC reagent for 30 minutes, and washed again in PBS for an additional 10 minutes. Sections were then developed with DAB substrate for 3 minutes, washed, and counterstained with hematoxilin. After dehydration and mounting, the slides were visualized using Leitz microscope equiped with digital image analysis apparatus.

a.6. 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.7. 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.

a. Results

b.1. cyclin D1 overexpression in DMBA-tumors

Our initial effort was to examine MDM2 and cyclin D1 gene expression in mammary tumors using RT-PCR. The rationale was that since MDM2 amplification has been documented in many human tumors including breast cancers (Deng et al., 1995; Fontana et al., 1994; McCann et al., 1995; Momand et al., 1998) and cyclin D1 appears to play a important role in mammary tumorigenesis (Bartkova et al., 1994; Musgrove et al., 1994; Sicinski et al., 1995; Wang et al., 1994; Weinstat-Saslow et al., 1995), it would be important to examine whether the environmental carcinogen DMBA-mediated mammary tumorigenesis is involved in MDM2 and cyclin D1. To do that, we performed RT-PCR to assess the gene expression of cyclin D1 and MDM2. GAPDH was also analyzed in parallel as control. As shown in Figure 1, cyclin D1 overexpression was evident in 5 out of 7 tumors (lanes 1-7) in comparison to the normal glands (lanes 8-10), whereas MDM2 appears to be no significant change.

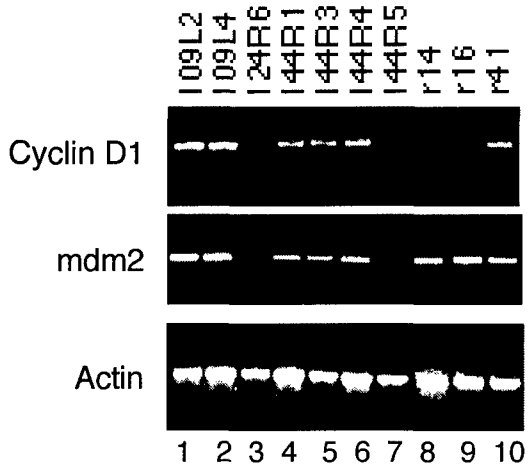


Figure 1. Overexpression of cyclin D1 in DMBA-induced mammary tumors. RT-PCR of cyclin D1, MDM2 and actin from DMBA-induced mammary tumors (lanes 1-7) or normal mammary gland (lanes 8-10). The PCR primers are specific for murine cyclin D1, murine MDM2, and actin, respectively, as described in Experimental Methods.

b.2. Immunobloting analysis on the expression of MDM2 and cell cycle proteins

Data from RT-PCR suggested that cyclin D1 was indeed overexpressed in tumors whereas MDM2 expression was not significantly altered. Therefore, we examined the protein expression levels by Western blot analysis. As shown in Figure 2, MDM2 overexpression was observed in all tumors in comparison to the normal glands derived from the same rat. The most striking difference, however, was the overexpression of p27 protein in the tumors. The prolonged exposure of same blot showed the basal level expression of p27 in normal glands (Figure 2, the third panel from top). In sharp contrast, the expression of p16, p53 and α-tublin appeared to be comparable in all samples.

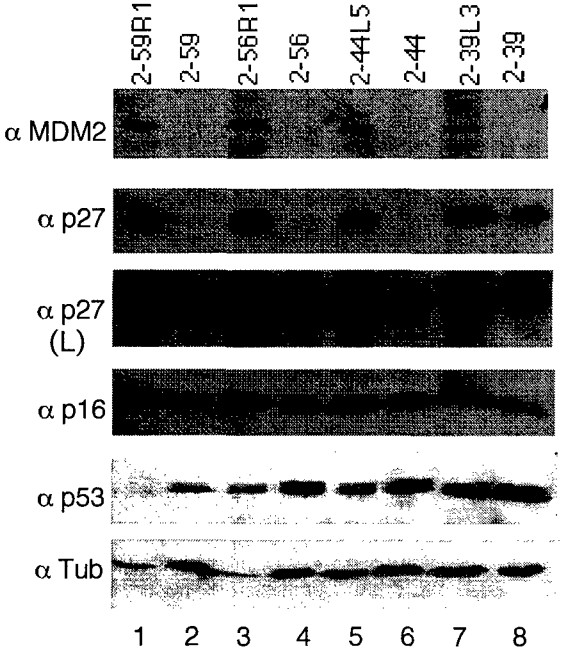


Figure 2 Protein expression in DMBA-tumors and normal mammary glands. Equal amount of the total protein (30 μg) from tumor samples (lanes 1, 3, 5 and 7) or normal mammary glands (lanes 2, 4, 6 and 8) were immunoblotted using antibody specific for MDM2 (SMP14, Santa Cruz), p27 (C19, Santa Cruz) and p16 (F12, Santa Cruz). To detect the basal level of p27 in normal mammary glands, the same blot was over exposed (the third panel form top).

b.3. Increased MDM2 protein expression in DMBA-induced mammary tumors

We then performed immunohistochjemical analysis to examine the expression of MDM2 in DMBA-induced rat mammary tumors and normal mammary glands. As shown in Figure 3, the nuclear staining of MDM2 was evident in the tumor sample compared to that of a normal mammary gland. This data further suggests that upregulation of MDM2 is an important characteristic of DMBA-rat mammary tumors and that MDM2 may play an important role in environmental pollutant-induced mammary tumorigenesis.

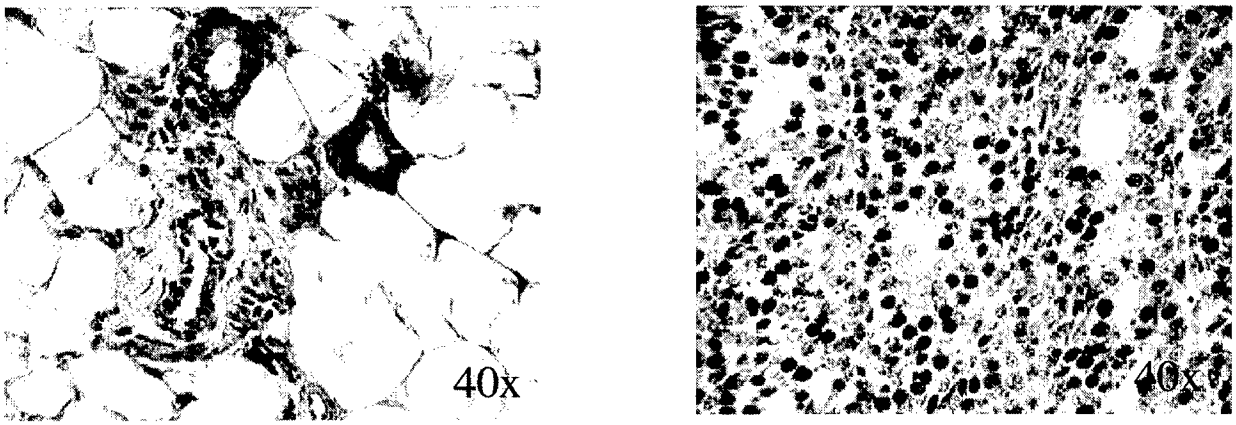


Figure 3. Increased MDM2 expression in DMBA-induced rat mammary tumors. Paraformaldehyde-fixed, paraffin embedded normal mammary glands (left) and tumors (right) from DMBA treated rats (18 weeks) were sectioned to 5 μm and affixed to slides. Slides were then subjected to immunohistochemical staining using a monoclonal antibody specific for MDM2 (2A10). Cells were counterstained with hematoxilin.

b.4. Northern blot analysis on p27 expression in tumors

Overexpression of p27 in tumors is evident as shown in Figure 2. However, it could be due to an increased transcription. To examine the possibility, we have performed Northern bolt analysis on p27. using a P32-labeled murine p27 cDNA as a probe. As shown in Figure 4, the steady-state mRNA levels of p27 were comparable, indicating that the transcription and/or mRNA stability is not the responsible for the overexpression of p27 in tumors.

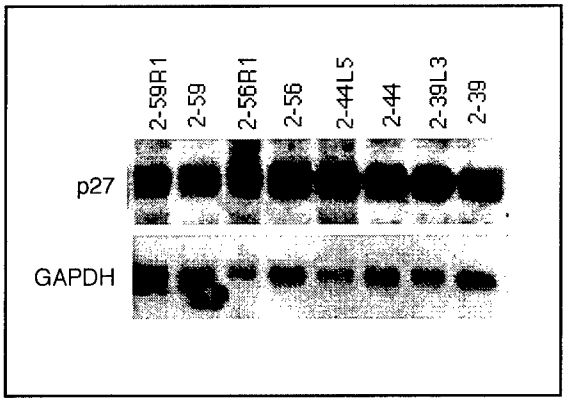


Figure 4. Upregulation of p27 expression is via a post-transcriptional mechanism(s). Total mRNA from tumors and normal glands were subjected to Northern blot analysis using a p32-labled p27 probe.

b.5. p27 protein expression during the time-course of DMBA-treatment

The overexpression of p27 is both striking and unexpected. Thus, we asked when p27 protein overexpression can be detected during the DMBA treatment. 50 μg of total proteins from various tissue samples was loaded per lane and separated on a 12% SDS-PAGE. Interestingly, we could not detect any significant alteration on p27 protein expression during the early stages (up to 9 weeks) of mammary tumorigenesis (Figure 5).

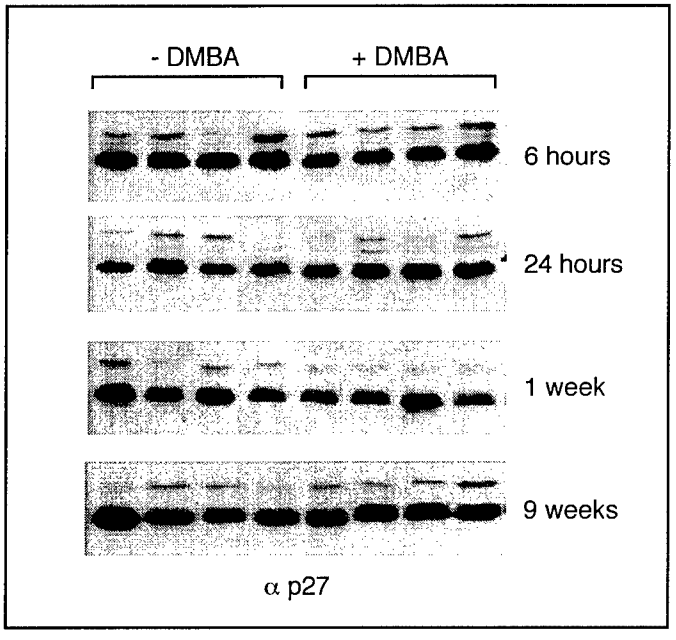


Figure 5. p27 protein expression during the time-course of DMBA-treatment.

b.6. Enhanced p27 protein expression in DMBA-induced mammary tumors.

We next performed immunohistochemical analysis of p27 protein expression in the DMBA-induced mammary tumors compared to the normal mammary glands. As shown in Figure 6, the specific nuclear staining of p27 was evident in the tumor samples. Under the same condition, there were barely any positive signals in the epithelial cells in the normal mammary glands. The high levels of p27 staining were particularly evident in the tumors 2-56R1 and 2-59R1. These data suggest that the alteration of p27 expression in tumor samples occurs in the mammary epithelial cells.

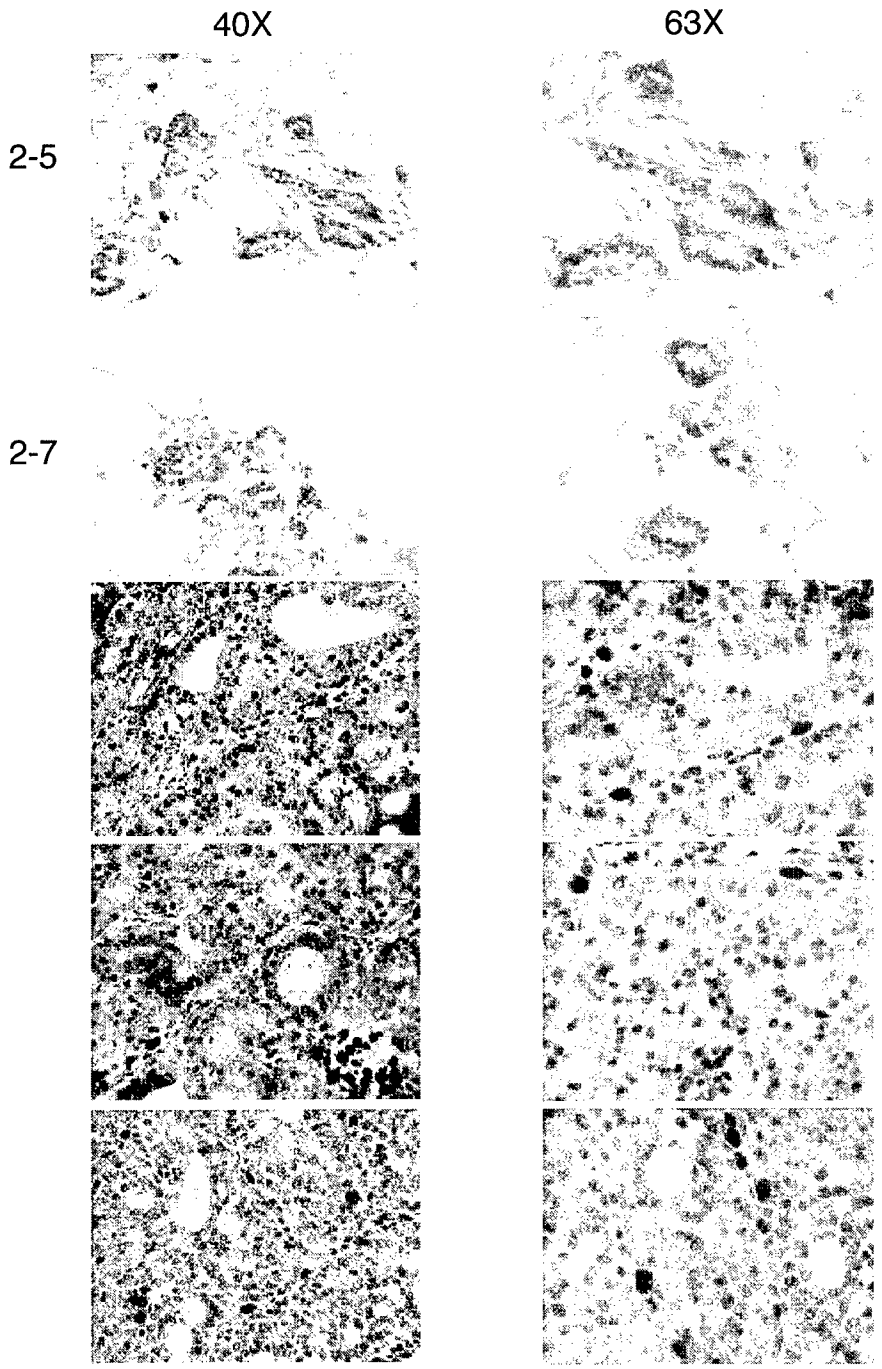


Figure 6. Increased p27 expression in DMBA-induced mammary tumors. Paraformaldehyde-fixed, paraffin embedded normal mammary glands (left) and tumors (right) from DMBA treated rats (18 weeks) were sectioned to 5 μm and affixed to slides. Slides were then subjected to immunohistochemical staining using a rabbit polyclonal antibody specific for p27 (C-19). Cells were counterstained with hematoxilin

b.7. 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 7). 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 8A). 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 8B). Both p53 and p21 appear to be up regulated albeit at much less extent (Figure 8B).

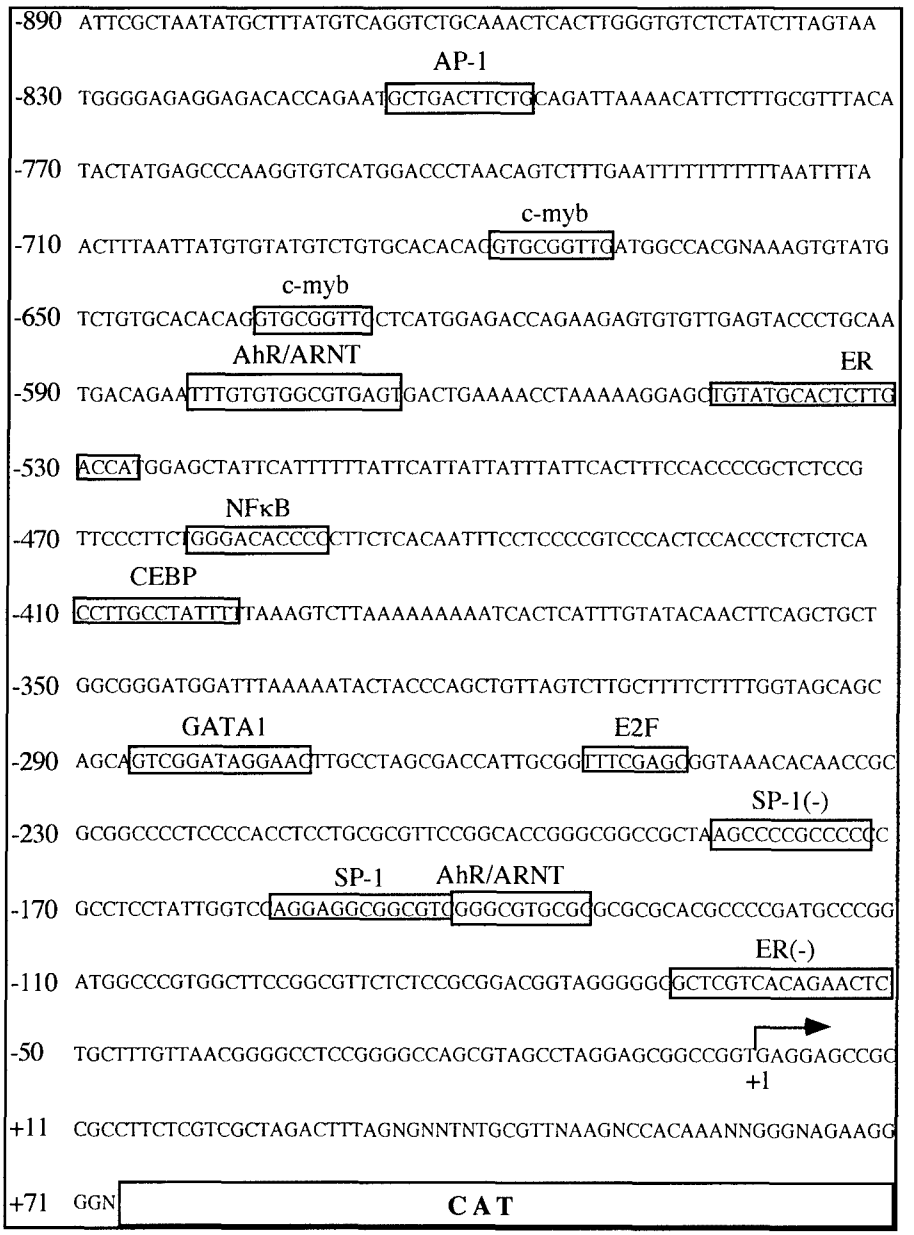


Figure 7. Some putative regulatory elements in the 5' UTR of human MDM2 gene. The putative regulatory elements match 100% with the core sequences and 80% or above the matrix consensus sequence (Quandt et al., 1995)

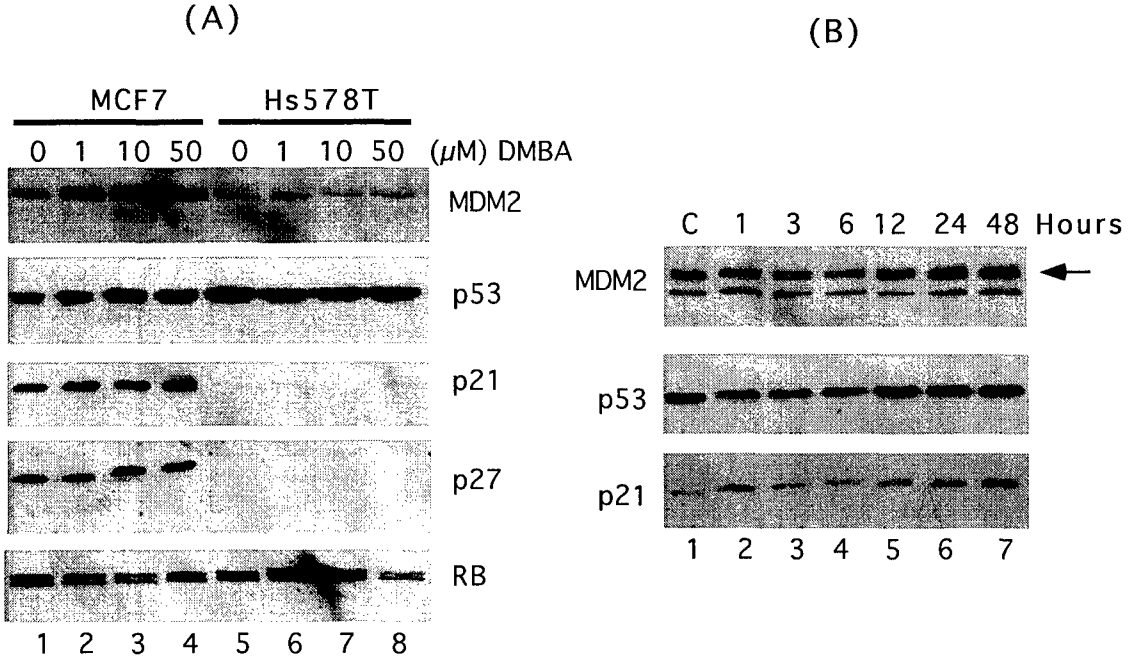
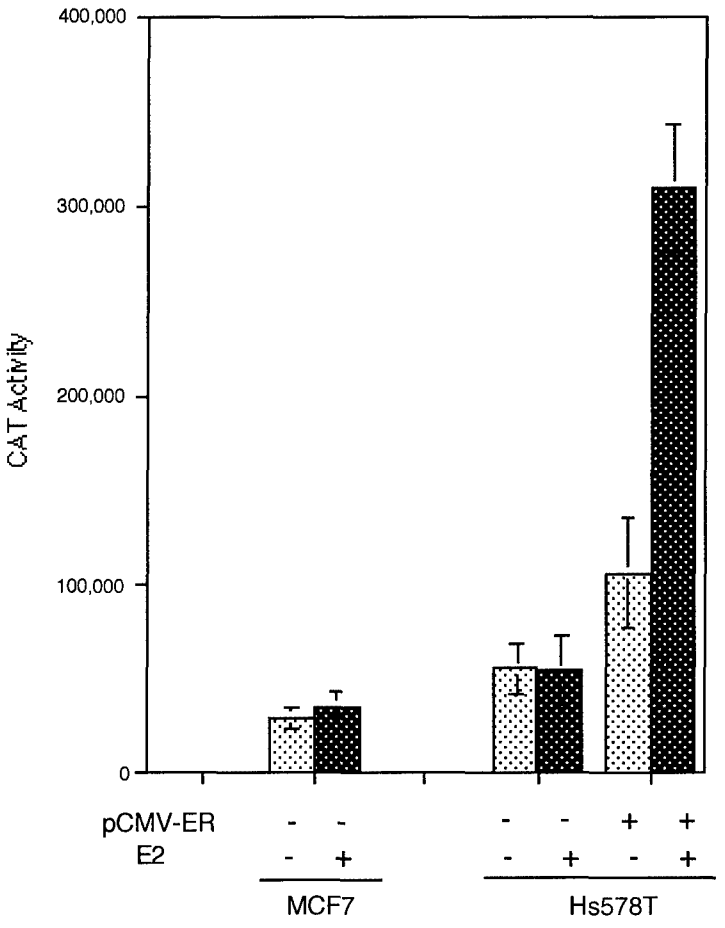


Figure 8. DMBA activates MDM2 expression in MCF7 cells in a dose- and time-dependent manner. Thirty to fifty μg of total protein was loaded per lane. The membrane was with probed with antibody (from Santa Cruz) specific for p53 (DO1), RB (IF8), p27 (C-19) and p21 (Pharmingen). Specific proteins were visualized using an ECL procedure (Amersham).

b.8. The effect of ER on MDM2 CAT activity in breast cancer cell lines



We next examined whether the 5' UTR in the MDM2-CAT reporter contains a functional element which can respond to the estrogen treatment. As shown in Figure 9, E2-treatment on the transfected MCF7 (an ER+ cell line) or Hs578T (an ER- cell line) did not increase the activity of MDM2-CAT reporter (Figure 9) whereas it dramatically increase an ER-reporter (data not shown). However, co-transfection of an expression ER construct in Hs578T cell significantly increase the MDM2-CAT activity, suggesting that the 5' UTR of MDM2 could functionally respond to ER. Whether overexpression of ER in MCF7 cells also increases MDM2-CAT reporter activity is not known.

Figure 9. The effects of E2 and overexpression of ER on the MDM2-CAT reporter activity in both ER+ and ER- cell lines.

b.9. 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 10, 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.

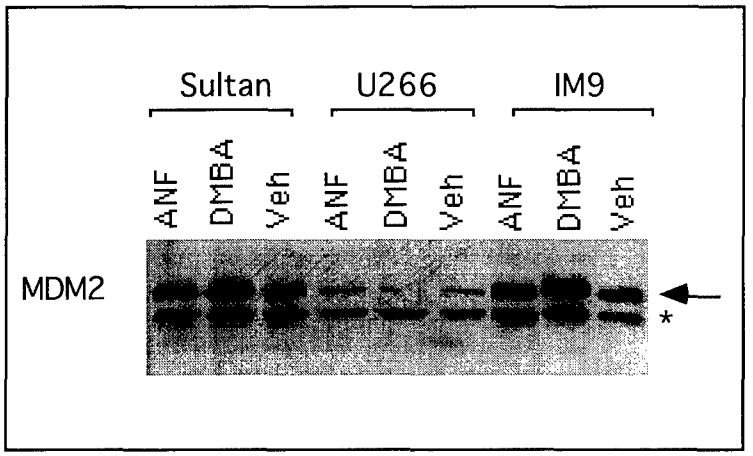


Figure 10. Expression of MDM2 correlates with AhR. 50 μg total protein was loaded per lane. Western blot analysis was performed using MDM2-specific antibody (2A10). MDM2 protein is indicated by an arrow. A non-specific protein is also indicated by an asterisk.

b.10. 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 11). 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 11, 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 11, lane 4), but not by the cold mutant oligo (P1m) (Figure 11, lane 5). DMBA-treatment of MCF-7 cells appeared to lead an increase of at least one specific protein-DNA complex (Figure 11, 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 7), 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 11, pane B, lane 2) or P3 (Figure 11, panel B, lane 3) oligo as probe.

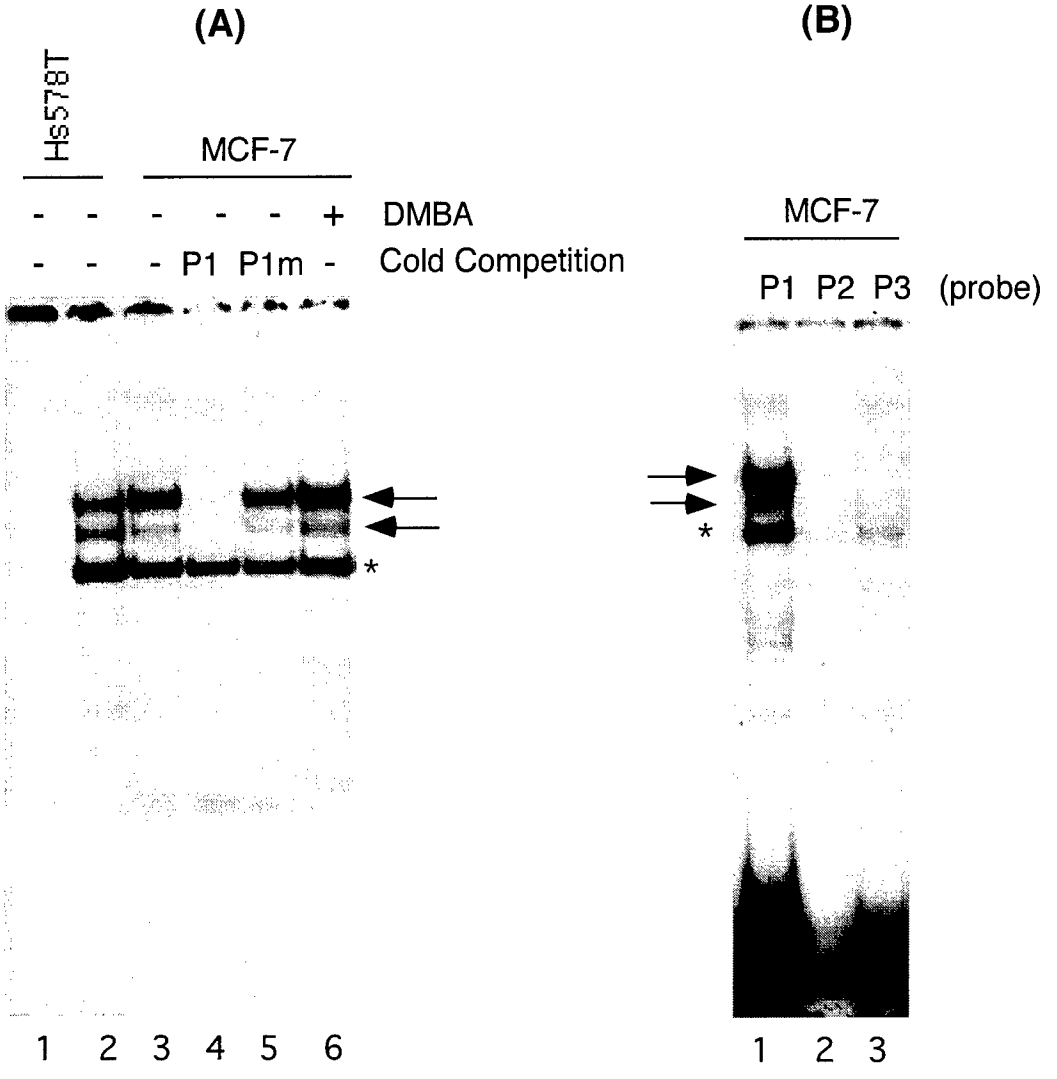


Figure 11. The AhR/ARNT site (-581/-566) on the MDM2 5' UTR binds to specific cellular proteins. Specific protein-DNA complexes are indicated by arrows whereas a non-specific protein is indicated by an asterisk.

b.11. Upregulation of MDM2 and p27 in breast cancer cell lines

Since the expression of MDM2 and p27 is elevated in carcinogen-induced mammary tumors, we wondered whether this phenomenon could be recapitulated in human breast cancer cell lines. We therefore examined the expression of MDM2 and p27 in four cell lines derived from human breast cancers (MCF-7, T47D, Hs578T, and MBA-MD-231) and compared to that of untransformed human breast epithelial MCF-10F cells. As shown in Figure 12, MDM2 protein levels in cancer cell lines were significantly higher compared to MCF-10F cells (lanes 2-5 versus lane 1). Interestingly, p27 expression was also significantly higher in cancer cells compared to MCF-10F cells.

In view of the observation that the estrogen receptor (ER) plays a critical role in human breast cancer, it is interesting to note that the p27 expression is significantly higher in ER positive MCF-7 and T47D breast cancer cells compared to that in ER negative cell lines Hs578T, MBA-MD-231, and MCF-10F. Thus, this data indicates that MDM2 and p27 is not only elevated in carcinogen-induced rat primary mammary tumors, but also in human breast cancer cells.

Given that MDM2 overexpression is apparent in the DMBA-induced mammary tumors and in human breast cancer cell lines, we considered whether up-regulation of MDM2 by DMBA may function to block normal p53 activation, in the course of tumorigenesis. Therefore, we examined the expression of MDM2, p53, and p21 in ER+ MCF-7 cells and ER- Hs578T after treatment with increasing doses of

DMBA. As demonstrated in Figure 13, DMBA induced a dose-dependent up-regulation of MDM2 in MCF-7 cells, but not in Hs578T cells (lanes 1-4 versus 5-8), with maximum stimulation occurring at 10 μM DMBA. Importantly, DMBA activated p53 and its downstream target protein p21 in MCF-7 cells, but not in Hs578T cells. Given the fact that MCF-7 cells contain wild type p53 protein, whereas Hs578T cells carry a gene which encodes a non-functional p53 protein (V157F) (Nieves-Neira and Pommier, 1999), this data suggests that activation of MDM2 in response to DMBA may requires p53.

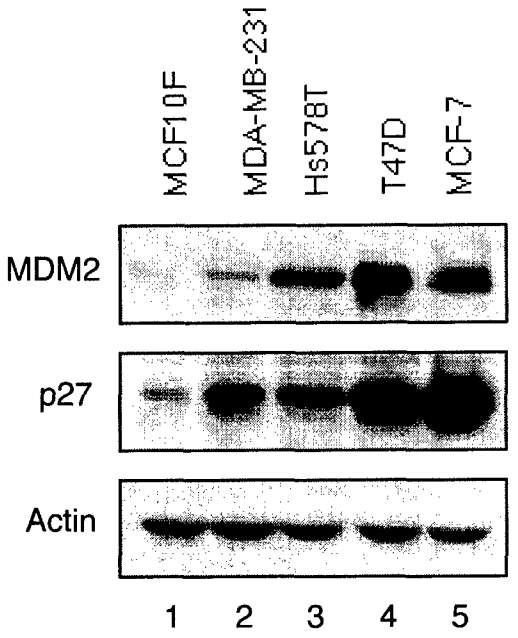


Figure 12. Upregulation of MDM2 and p27 in breast cancer cell lines. Sub-confluent untransformed human breast cells (MCF-10F) and human breast cancer cell lines (MBA-MD-231, Hs578T, T47D, MCF-7) were lysed in EBC buffer. Fifty μg of total protein was loaded, separated on a 10% SDS-PAGE and transferred to a PVDF membrane. Western blot analyses were performed using antibodies specific for p27 (C-19), MDM2 (SMP-14), or actin (C-11).

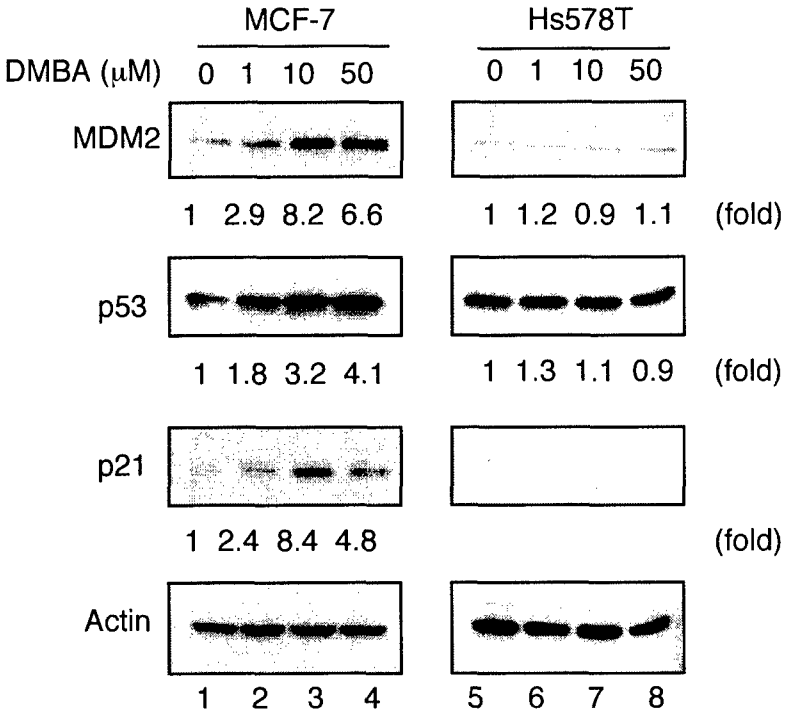


Figure 13. DMBA induces the expression of p53, MDM2, and p21 in MCF-7, but not in Hs578T breast cancer cells. Sub-confluent MCF-7 and Hs578T cells were treated with DMSO control or the indicated dose of DMBA. Twenty-four hours post-treatment, cells were washed twice with PBS and lysed in EBC buffer. Fifty μg of total protein was loaded, separated on a 10% SDS-PAGE and transferred to a PVDF membrane. Western blot analyses were performed using antibodies specific for p53 (DO-1), MDM2 (SMP-14), p21 (SXM 30), or actin (C-11). Quantitation was performed using Labworks Image Acquisition and Analysis Software V. 4.0.0.8 (UVP Bioimaging Systems), the expression of MDM2, p53, and p21 normalized to actin, and the expression level of untreated samples arbitrarily set to 1.0.

b.12. Time-dependent up-regulation of p53, MDM2, and p21 upon DMBA treatment.

To extend our observations that DMBA can activate p53, MDM2, and p21 in MCF-7 cells, we examined the time-dependent effect of DMBA on their expression. Treatment with 10 μM DMBA induced a clear up-regulation of all three proteins after 12 hours (Figure 14, lane 5 versus lane 1), and protein expression remained high at both 24 and 48 hours (lanes 6-7). This suggests upregulation of p53, MDM2, and p21 is a relatively late event. Considering that activation of gene expression by DMBA via conjugation with AhR/ARNT occurs quite rapidly, this data also suggests upregulation of MDM2, p53, and p21 is not through direct transcriptional stimulation, but likely involves initiation of DNA damage by DMBA. Therefore, in view of the fact that MDM2 is a transcriptional target of p53, it seems plausible that the observed activation of MDM2 represents one component of a normal p53-dependent response to genotoxic stress.

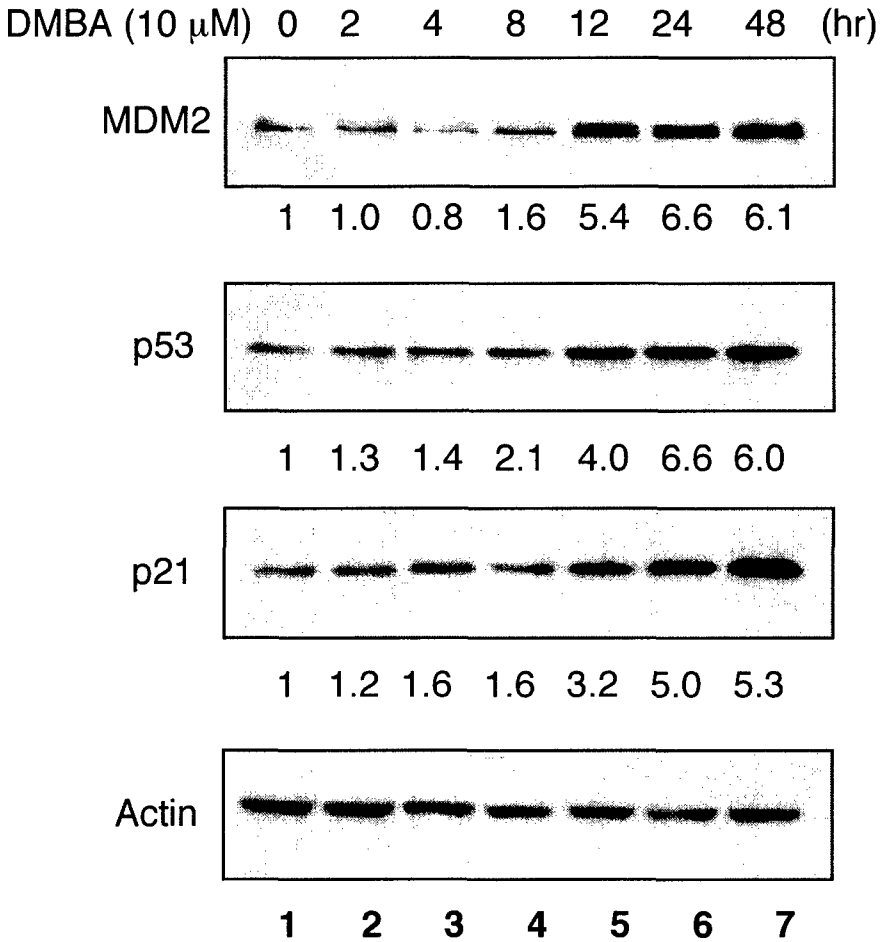


Figure 28. Time-dependent up-regulation of p53, MDM2, and p21 upon DMBA treatment. Sub-confluent MCF-7 and Hs578T cells were treated with DMSO control or 10 μM DMBA. Following treatment, cells were washed twice with PBS and lysed in EBC buffer at the times indicated. Fifty μg of total protein was loaded and separated on a 10% SDS-PAGE and transferred to a PVDF membrane. Western blot analysis was performed using antibodies specific for p53 (DO-1), MDM2 (SMP-14), p21 (SXM 30), or actin (C-11). Quantitation was performed using Labworks Image Acquisition and Analysis Software V. 4.0.0.8 (UVP Bioimaging Systems), the expression of MDM2, p53, and p21 normalized to actin, and the expression level of untreated samples arbitrarily set to 1.0.

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.

Because MDM2 is a critical negative regulator of p53, the enhanced MDM2 expression represents an alternate strategy for abrogation of p53 function, an important step in oncogenesis. Our data demonstrates up-regulation of MDM2 protein expression in DMBA-rat mammary tumors by Western blot and immunohistochemistry, suggesting a role for MDM2 in environmental carcinogen-induced of breast cancer. Interestingly, analysis of the p53 status of rat mammary tumors induced by DMBA reveals infrequent mutations of p53 (Kito et al., 1996), lending support to the notion that alternate means of blocking p53 activity may be critical in this model of carcinogenesis. Our observation of MDM2 overexpression in DMBA-induced rat mammary tumors is consistent with this model.

Examination of the expression of both p27 and MDM2 in human breast cancer cell lines and untransformed MCF-10F mammary epithelial cell lines revealed high levels of MDM2 expression in cancer cell lines, consistent with the notion that abrogation of p53 function through enhanced MDM2 expression may represent an important step in tumorigenesis. Furthermore, our data confirms the observation that MDM2 expression is higher in ER+ cell lines, consistent with a previously reported possible link between the ER and MDM2 (Sheikh et al., 1993).

Substantial evidence exists supporting the notion that there is significant crosstalk between ER and AhR pathways, particularly a cooperative effect of ovarian hormones and polyaromatic hydrocarbons in carcinogenesis (Lucier et al., 1991; Vickers et al., 1989). Consistent with this model, we show DMBA treatment results in a dose dependent increase in MDM2 expression in ER+ MCF-7, but not ER- Hs578T cells. Thus, chronically elevated levels of the oncoprotein may represent a critical mechanism for abbrogation of p53 tumor suppressor function later in oncogenesis.

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. 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.

Alteration of p27 expression in DMBA-rat mammary tumors

In this study, we find dramatically enhanced p27 protein expression in DMBA-rat mammary tumors, indicated by both Western blot and immunohistochemical staining. Up-regulation of p27 appears to be a late event during mammary tumorigenesis, suggesting that the alteration of expression may not involved in the initiation of mammary tumorigenesis, but perhaps is important for the proper maintenance of the established lesion.

In addition, our data shows that p27 mRNA expression is not significantly altered in DMBA-rat tumors compared to that of control mammary glands, suggesting that the up-regulation of p27 protein observed in the tumors may occur via post-transcriptional mechanism. It is well documented that alteration of p27 protein stability comprises a critical regulatory mechanism.

The finding that p27 protein levels are elevated in total protein extracts derived from DMBA-induced mammary tumors versus normal mammary glands raises the question of whether differences in the overall tissue composition could account for these differences, since the proportion of epithelial cells is significantly higher in tumor samples than in normal mammary glands, which are also composed of adipose, connective, and muscle tissues. Thus, it is possible that the observed alteration in p27 expression may simply reflect the apparent expansion of the total number of epithelial cells. Our data, however, shows that the DMBA-rat tumor samples exhibit substantially enhanced p27 immunoreactivity in a large number of the cells compared to normal controls, indicating that p27 expression is upregulated in these tumors. In agreement with our findings in DMBA-induced rat mammary tumors, we also observe increased expression of p27 protein in human breast cancer cell lines compared to untransformed MCF-10F cells. This suggests increased expression of p27 also occurs in human cancers as well, although the mechanism remains unclear.

One possible explanation for this phenomenon might be that increased p27 expression results in reduced susceptibility to apoptosis. The development of a malignant tumor is characterized by the balance between the rapid proliferation of the transformed cells and the high degree of cell attrition due to apoptosis. A modest increase in survival, therefore, can be manifest in a dramatic increase in the overall rate of tumor growth. Indeed, several studies have shown that increased expression of p27 promotes survival in several systems. Apoptosis in FRTL-5 thyroid cells is accompanied by a decrease in p27, coupled with upregulation of c-myc (Carneiro et al., 1998). Increased adhesion signaling has been shown to upregulate p27, which in turn protects cells from drug-induced apoptosis (La Croix and Russo, 1996; St Croix and Kerbel, 1997). Additionally, p27 expression has been shown to be important in enhancing cell survival in neuronal cells (Park et al., 1997) and during inflammatory injury response (Ophascharoensuk et al., 1998). Moreover, p27 can block cyctochrome c release and prevent caspase activation in leukemia cells (Eymin et al., 1999). Interestingly, $p27^{-/-}$ fibroblasts are sensitive to apoptosis upon growth factor withdrawal (Hiromura et al., 1999), a phenotype which can be rescued by restoration of p27 expression or inhibition of cdk2 activity. However, several other reports have found that overexpression of p27 can trigger apoptosis after several days in culture (Craig et al., 1997; Katayose et al., 1997; Schreiber et al., 1999), suggesting that p27 may play a complex role in the regulation of apoptosis and the outcome may depend cell type, physiological conditions, growth factor availability, among others.

Another plausible explanation derives from the observation that p27 protein functions as an assembly factor for cyclin D/cdk 4/6 holoenzyme complexes. Several studies have clearly demonstrated that p27 only serves as an inhibitor of D-type cyclin complexes when overexpressed at high levels (Polyak et al., 1994; Sherr and Roberts, 1999; Toroshima and Hunter, 1994), and actually serves to activate the kinase at physiological stoichiometry (Blain et al., 1997; LaBaer et al., 1997). Indeed, mice lacking p27 exhibit reduced cyclin D/cdk activity, the activity of which is further reduced in p27/p21 double-null mice (Cheng et al., 1999). Therefore, considering the critical role overproduction of cyclin D1 in breast cancer (Barnes and Gillett, 1998), it is possible that expansion of proliferating mammary epithelial cells require increased expression of p27 for proper assembly of cyclin D/cdk complexes. Consistent with this model, the expression of cyclin D1 is significantly enhanced in DMBA-rat tumors. Interestingly, Fredersdorf et al show dramatically increased p27 expression in several highly proliferative human breast cancer cell lines, correlating with increased cyclin D1 expression (Fredersdorf et al., 1997), in agreement with our analysis of p27 expression in breast cancer cell lines. A similar pattern is also seen in low-grade primary breast tumors (Fredersdorf et al., 1997). Thus, up-regulation of p27 in DMBA-rat tumors may be due to the increased expression of cyclin D1, allowing for proper activation of cdk complexes and promoting cell cycle progression of the developing tumor. Further investigation, however, is needed to appropriately address this hypothesis.

KEY RESEARCH ACCOMPLISHMENS

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

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

b. 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.

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

d. 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.

e. We demonstrate that p27 is over expressed in the environmental carcinogen-induced rat mammary tumorigenesis, which is novel and significant in understanding the etiology of breast cancer.

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.

SA Murray and Z.-X. J. Xiao. OVERPRODUCION OF MDM2 IN DMBA-INDUCED RAT MAMMARY TUMORS AND REGUALTION OF MDM2 BY ESTROGEN. Department of Defense Conference "Era of Hope", June, 2000, Atlanta. Abstract in appendices #1

SA Murray and Z.-X. J. Xiao. Overproduction of MDM2 in DMBA-induced Rat Mammary Tumors and Effects of the Estrogen Receptor and the Aryl Hydrocarbon Receptor on MDM2 Expression. " Russek Student Day". Poster presentation. Appendices #2.

SA Murray, H. Zheng, L. Gu and Z.-X. J. Xiao. p21CIP1 is essential in IGF-1 survival function upon UV irradiation. Revised manuscript submitted.

SA Murray and Z.-X. J. Xiao. Altered expression of MDM2 and p27 in DMBA-induced rat tumors. Manuscript in preparation.

Degree obtained

SA Murray was partially funded by this award and it is expected that he will obtain his Ph.D. degree in June, 2002.

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.

List of personnel receiving pay from the research effort

Zhi-Xiong Jim Xiao, Ph.D.
Stephen A. Murray, Ph.D. student, expected Ph.D. degree in June, 2002.
Wei Chen, Master degree student.
Fan Xiang, technician.

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. 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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Appendices 1. Meeting abstract for "Era of Hope"

Meeting Abstract for "Era of Hope" (June 8-12, 2000, Atlanta)
Department of Defense
Breast Cancer Research Program

OVERPRODUCION OF MDM2 IN DMBA-INDUCED RAT MAMMARY TUMORS AND REGUALTION OF MDM2 BY ESTROGEN
RECEPTOR AND AROMATIC HYDROCARBON RECEPTOR

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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.

Appendices #2. Meeting abstract for "Russek Student Achievement Day", February, 2000.

Overproduction of MDM2 in DMBA-induced Rat Mammary Tumors and Effects of the Estrogen Receptor and the Aryl Hydrocarbon Receptor on MDM2 Expression

Stephen Murray, Department of Biochemistry, Advisor: Dr. Jim Xiao

A growing body of evidence indicates that exposure to environmental pollutants such as polycyclic aromatic hydrocarbons (PAH) contributes to the recent increase in breast cancer incidence and mortality. The molecular mechanisms by which these carcinogens induce breast tumors are poorly understood. 7,12-dimethylbenz(α)anthracene (DMBA) is a member of the PAH family that can potently and selectively induces mammary tumor formation in female Sprague-Dawley rats. Estrogen is indispensable for the DMBA-mediated mammary tumorigenesis. Since MDM2 is overproduced in many human tumors and cancers including breast cancer, we hypothesize that DMBA and estrogen receptor (ER) cooperate in activation of protooncogene *Mdm2* in the DMBA-rat mammary tumor model. We have analyzed RNA and protein levels from mammary glands collected from DMBA- or vehicle-treated control animals at 6 hours, 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, p21 and RB. In addition, we have examined a number of human cancer cell lines and found that MDM2 expression closely correlates with the expression of the aromatic hydrocarbon receptor (AhR) and ER. Additionally, 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. These results have important implications for environmental carcinogens on the etiology of breast cancer.

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

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