Estradiol Stabilizes p53 Protein in Breast Cancer Cell Line, MCF-7

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Overexpression of the oncoprotein MDM2, an important regulator of the p53 tumor suppressor protein, is often observed in breast cancer tissues and cell lines, particularly in those which express estrogen receptor α (ERα). In MCF-7 breast cancer cell line possessing wild-type p53, ERα, and overexpressing MDM2, p53 accumulation was stimulated by 17β-estradiol (E2) in a concentration-dependent manner. On the other hand, E2 caused no change of the expression of p53 mRNA, indicating that E2 affects p53 at the post-transcriptional level. To analyze the mechanism of p53 accumulation by E2, the stability of p53, ERα and MDM2 proteins was analyzed in the presence of cycloheximide under an E2-supplemented or -depleted condition. E2 significantly extended the half-life of p53 protein, but shortened that of ERα in MCF-7 cells. E2 significantly decreased the stability of p90(MDM2) and p60(MDM2) in MCF-7. Interestingly, E2 increased the ratio p60(MDM2)/p90(MDM2) inversely proportionally to the degradation of p53. These results suggest that the ratio of the two MDM2 proteins, p90(MDM2) and p60(MDM2), may affect the accumulation of wild-type p53 protein in response to E2.

Key words: Breast cancer — p53 — MDM2 — Estrogen receptor

Since the mdm2 gene is frequently amplified in some types of cancers, including osteosarcoma,1—4 and shows oncogenic activity in NIH3T3 cells when exogenously introduced,5 it is considered to be an oncogene. The human mdm2 gene encodes a polypeptide consisting of 491 amino acids that contains a binding domain for the tumor suppressor p53.5—8 MDM2 binds to the N-terminal region of p53 and inhibits its transcriptional activity by concealing the transactivation domain.1, 6 In addition, MDM2 promotes rapid degradation of p53 via the ubiquitin-proteasome pathway.9, 10 In contrast, the mdm2 gene is transcriptionally regulated by p53. Therefore, MDM2 is thought to be a negative feedback regulator of this tumor suppressor.6, 7 The expression of p53 is strictly regulated, mainly by MDM2. In turn, p53 is a transcription factor of MDM2, so this negative feedback loop is very important for cell integrity.6, 7 In various species and tissues, MDM2 showed various molecular weight patterns in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), for example, 90, 76/74 and 60 kDa, although the nature of these proteins is still unclear.11—14 In MCF-7 cells, p90(MDM2) was dominantly expressed but p60(MDM2) was also detected by western blotting with a specific antibody which recognizes MDM2.15

Although mdm2 gene amplification is uncommon in breast cancers, the level of its mRNA and/or protein is up-regulated in about 30—40% of breast cancer specimens.16—18 Interestingly, there was a positive correlation between the levels of MDM2 and estrogen receptor α (ERα) in breast cancer specimens and cell lines.11, 19 In contrast to ERα-negative cell lines, all ERα-positive cells examined have expressed elevated levels of mdm2 mRNA.11

The expression of wild-type p53 correlates well with that of ERα in human breast cancer specimens.20, 21 Moreover, p53 down-regulated the transcriptional activity of ERα22 and 17β-estradiol (E2) induced functional inactivation of p53 in MCF-7 cells,23 suggesting an intimate interaction between p53 and ERα in human breast cancer.

These observations prompted us to look for the functional interactions among p53, MDM2 and ERα in human breast cancers. We have already reported that E2 induced accumulation of p53 protein15 and that MDM2 enhanced the transcriptional activity of ERα in MCF-7 cells.24

In the present study, we investigated the mechanisms by which p53 accumulation occurred in response to E2, and found that E2 affected the stability of wild-type p53 protein.

MATERIALS AND METHODS

Cell culture Human mammary tumor cell lines (MCF-7, T47-D and MDA-MB-231) were cultured in RPMI1640 medium (Gibco BRL, Grand Island, NY) supplemented

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with 10% fetal bovine serum (Gibco BRL), 1 nM E2 (Wako Pure Chemical Industries, Osaka), 2 mM L-glutamine, 5 units/ml penicillin and 5 μg/ml streptomycin (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere of 5% CO2 in air.25 Wild-type p53 is expressed only in the MCF-7 cell line. T47-D and MDA-MB-231 cell lines possess missense point mutations in the p53 gene at codons 194 (CTT→TTT) and 280 (AGA→AAA), respectively.26

**Protein stability assay** Fifty thousand cells were cultured for 48 h in phenol red-free DMEM (Dulbecco’s modified Eagle’s medium; PRF-DMEM, Gibco BRL) containing 10% FBS (fetal bovine serum) stripped of steroids by absorption on dextran-coated charcoal (DCC-FBS) in 6-cm culture dishes. Cells were incubated in the medium supplemented with or without 10 nM E2 for 24 h. To inhibit protein synthesis, cycloheximide (30 μg/ml) was added to the conditioned medium and cells were then harvested in lysis buffer (1% Triton X-100, 50 mM NaCl, 25 mM Hepes (pH 7.4), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin) at the indicated time points. Lysates were scraped and transferred into microtubes, then centrifuged at 12,000g for 10 min. The supernatants were used as cell extracts.

**Western blot analysis** Protein contents were determined with a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Twenty micrograms of proteins was separated by 9% SDS-PAGE and subsequently electrophoretically transferred onto PVDF membrane (Millipore, Bedford, MA) using transfer buffer (25 mM Tris, 192 mM glycine). To assess the quality of electrophoretic transfer, prestained SDS-PAGE standards (Bio-Rad) were used. The membrane was incubated for 24 h in blocking buffer (2% skimmed milk, 0.1% Tween-20 in 20 mM Tris-buffered saline pH 7.5; TBS-T) at 4°C. The membrane was washed with TBS-T, and incubated with the respective primary antibody for 2 h. p53 was detected by 0.1 g/ml Ab-6 mouse monoclonal antibody (Oncogene Research Products, Cambridge, MA), which recognized an amino-terminal epitope mapping between amino acid residues 11 and 25 of human p53 and reacted with mutant as well as wild-type p53.11,27 ERα and MDM2 were detected with 1 μg/ml D-12 mouse monoclonal antibody and 1 μg/ml SMP14 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Membranes were washed with TBS-T, then incubated with secondary horse-radish peroxidase-conjugated anti-mouse-immunoglobulin G (IgG) or anti-rabbit-IgG (Amersham, Buckinghamshire, UK), and probed by an ECL chemiluminescence technique (Amersham) according to the manufacturer’s recommendations. The density of each band was quantified by a densitometer (Atto Densitograph, Tokyo).

**RNA isolation and northern blot analysis** Cultured cells in a 6-cm dish were mixed with 1 ml of RNA extraction reagent, Isogen (Wako Pure Chemical Industries). Total RNA was extracted according to the manufacturer’s instructions. Twenty micrograms of total RNA was subjected to electrophoresis in 1% agarose/formaldehyde gel in 3-(N-morpholino)propanesulfonic acid (MOPS)/sodium acetate buffer. After electrophoresis, RNA was transferred onto a nylon membrane by capillary elution transfer. After prehybridization for 4 h at 42°C in hybridization buffer containing 50% formamide, 1% SDS, 1 M NaCl, 50 mM Tris/HCl (pH 7.0), 0.1 mg/ml denatured salmon sperm DNA, Denhart’s solution (0.1% bovine serum albumin, 0.1% Ficoll 400 and 0.1% polyvinylpyrrolidone), the membrane was hybridized with 32P-labeled probes for 16 h at 42°C. The probe used was synthesized by the PCR amplification technique using primers 5′-GCC GAA TTC AGA CCT ATG GAA ACT ACT TCC-3′ (sense) and 5′-ATA AAG CTT GCT GGT GTT GTT GGG CAG TGC-3′ (anti-sense) based on the human p53 cDNA sequence.28 The membrane was then washed twice in 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate, pH 7.0) containing 0.1% SDS at 45°C for 30 min and exposed to Kodak XO mat AR film (Rochester, NH). The density of each band was measured with a densitometer (Atto).

**Statistical analysis** The statistical significance of differences in the results was evaluated by means of one-factor ANOVA, and P values were calculated by the Bonferroni method. P values of less than 0.05 were considered significant.

**RESULTS**

**E2 enhanced p53 accumulation in a concentration-dependent manner** To examine whether the E2 signal mediated by ERα alters the expression level of p53 protein, we first performed western blotting of various cell lysates of MCF-7 cells grown under the indicated concentrations (Fig. 1A). Expression levels of p53 protein were significantly increased by E2 in a concentration-dependent manner in MCF-7 cells (Fig. 1B).

Then we examined whether the p53 accumulation in response to E2 occurred at the transcriptional level. As shown in Fig. 2, p53 mRNA expression, as assessed by northern blotting, was not significantly affected (P>0.01, compared with E2 (−)) by E2, strongly indicating that the increase of p53 protein in response to E2 is due to post-transcriptional control.

**E2 increased p53 protein stability in MCF-7 cell line** To investigate whether p53 protein is stabilized by E2, protein stability assay was performed in the same cell line. The relative expression level of p53 was monitored after addition of cycloheximide, which inhibits protein synthesis (Fig. 3). As was reported previously,16 the expression level of p53 protein was enhanced by E2 after culture for 48 h (compare Fig. 3A, lanes 1 and 6). In MCF-7 cells under an E2-depleted condition, p53 was very rapidly
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degraded; 70% of the protein was degraded within an hour. This was in accord with a previous report that p53 is a short-lived nuclear protein with a half-life of ~5–30 min in the stable state in most cell types studied. However, 10 nM E2 significantly delayed p53 degradation and prolonged the p53 half-life to approximately 90 min (Fig. 3B).

Next, we explored the change of stability of ERα protein in response to E2 in MCF-7 cells. The ERα protein level was unchanged within the first 2 h after inhibition of protein synthesis, and thereafter gradually decreased (Fig. 3C). Moreover, E2 significantly decreased the ERα stability.

E2 enhanced the ratio of p60(MDM2) to p90(MDM2)

Next, we explored the stability of MDM2 protein, an E3 ubiquitin ligase of p53, in response to E2, taking the composition of different molecular sizes of MDM2 into account (Fig. 4A). The antibody (SMP14) used here mainly recognized three protein bands corresponding to 90, 76 and 60 kDa. The 76 kDa band could not be detected by another antibody (IF2), as reported previously and its density was not significantly changed during incubation. Therefore, the changes of the two bands corresponding to 90 kDa, referred to as p90(MDM2) and 60 kDa, referred to as p60(MDM2) were analyzed in the present study. The major form of MDM2, p90(MDM2) gradually decreased (Fig. 4B). In the presence of E2, the degradation of p90(MDM2) was accelerated, indicating that E2 affects its protein stability. Under the same conditions, p53 protein was degraded more slowly than in the absence of E2 (Fig. 3B). This finding is consistent with the previous report that p90(MDM2) promotes the degradation of p53. Namely, acceleration of p90(MDM2) protein degradation by E2 was associated with increased stability of p53 protein. On the other hand, the level of p60(MDM2), a minor form, was more stable for 8 h after the inhibition of protein synthesis in the absence of E2. Supplementation of E2 significantly accelerated the degradation of p60(MDM2), and increased the p60/p90 ratio, especially after 4 h (Fig. 4D).

The stability of mutant p53 was not affected by E2 treatment in T47-D and MDA-MB-231 cell lines Next,
we examined the stability of mutant p53 using T47-D (ERα-positive) and MDA-MB-231 (ERα-negative) breast cancer cell lines. These cell lines possess missense point mutations in the DNA-binding domain of p53 gene at codons 194 and 280, respectively.26) The stability of p53 protein in these cells was greatly increased (Fig. 5), as compared with that in MCF-7 cells which possess wild-type p53. These results are consistent with the notion that the half-life of mutant p53 is longer than that of the wild-type.30, 31) Although T47-D cells express ERα protein, no significant difference in the stability of p53 protein was observed between E2-treated and -depleted cells (Fig. 5).
These results indicate that the stability of mutant p53 with a missense mutation at the core domain was not affected by E2 treatment, even in ERα-positive cells. In addition to p53, we also analyzed the protein stability of ERα and MDM2 in these cell lines. ERα was more rapidly degraded in the presence of E2 in T47-D cells (Fig. 5A), as observed in a variety of breast cancer cell lines.32) MDM2 levels in T47-D and MDA-MB-231 breast cancer cell lines were very low compared to that of the MCF-7 cell line, as reported.11) A longer exposure time (three to five times more) was necessary to detect p60(MDM2) as well as p90(MDM2) in both cell lines compared to MCF-7. However, E2 did not significantly alter the degradation of p90(MDM2) and p60(MDM2) in these cell lines.

**DISCUSSION**

It is well known that the MDM2 protein, which negatively regulates p53, is expressed at higher levels in ERα-positive breast cancers than in negative ones.10) We previously reported that stable transformants of MCF-7 cells bearing an mdm2-expressing vector showed increased numbers of colonies in a soft agar colony formation assay, compared with parental MCF-7 cells in the presence of E2.15) These results indicate that overexpressed MDM2 protein may provide a growth advantage to ERα-positive breast cancer. In addition, we have recently reported that MDM2 enhanced the transcriptional function of ERα through a direct interaction with ERα in a ligand-dependent manner.24) In the present study, we showed that wild-type p53 was accumulated in response to E2 in a concentration-dependent manner in MCF-7 cells (Fig. 1).

Here, we have analyzed the protein stability of p53, ERα and MDM2 to elucidate the mechanism of the p53 accumulation in response to E2 in human breast cancer cell lines. The level of p53 mRNA showed no significant changes regardless of E2 supplementation in MCF-7 cells (Fig. 2). Moreover, the protein stability assay of p53 in MCF-7 cells revealed that its half-life was extended by E2 treatment, with a statistical significance (Fig. 3). These findings suggested that p53 accumulation in response to E2 was not due to the transcriptional regulation of mRNA, but to a posttranslational modification, namely stabilization of the protein in the cells.

Since MDM2 is believed to promote the rapid degradation of wild-type p53 to maintain its low expression level,10) the change of MDM2 protein level was examined in the course of p53 degradation. MDM2 was detected as p60(MDM2) and p90(MDM2) with apparent molecular weights of 60 and 90 kDa, respectively, by western blot analysis in MCF-7 cells (Fig. 4A). Although the molecular variety could be due to sumoylation13) or alternative splicing,14) no definitive explanation is yet available. While p90(MDM2) showed a decrease, p60(MDM2) was well
maintained after addition of cycloheximide under an E2-depleted condition for 4 h. Since protein synthesis was inhibited, the maintenance of p60(MDM2) should be caused by a post-translational modification. The mechanism of formation of p60(MDM2) is still unclear. However, Chen et al. reported that MDM2 was cleaved by an caspase 3-like enzyme, leading to the accumulation of MDM2 with apparent molecular weight 60 kDa, with loss of 361 amino acids. This truncated form of MDM2 could bind to p53, but failed to promote p53 degradation due to the lack of the ring finger domain at the C-terminus, thereby apparently stabilizing p53 in a dominant-negative fashion. We constructed an expression vector for this truncated form and introduced the vector into COS-7 cells to perform western blot analysis. We found a slight but distinct difference of migration between the truncated form and naturally produced p60(MDM2) (data not shown).

In our experiment, E2 caused an acceleration of the degradation of both p90(MDM2) and p60(MDM2) (Fig. 4B). The ratio of p60/p90 showed a time-dependent increase, suggesting that the proportion of p60(MDM2) to p90(MDM2) may be important for wild-type p53 stabilization. Both p60(MDM2) and p90(MDM2) were detected by the same specific monoclonal antibody that recognized the N-terminus of MDM2, indicating that at least the N-terminus of p60(MDM2) was conserved. In addition to these findings, we have already reported that MDM2 physically interacts with ER\(\alpha\), leading to increased levels of estrogen-dependent transcription. Taking all the results together, we propose the following mechanism. In ER\(\alpha\)-positive and wild-type p53-expressing breast cancers, E2 prolongs wild-type p53 protein stability, causing it to accumulate in the nucleus and stimulating transcription of target genes including mdm2. The overexpression of MDM2 enhances the transactivation activity of ER\(\alpha\), leading to cell proliferation. Therefore, the MCF-7 ER\(\alpha\)-positive breast cancer cell line acquires a remarkable growth advantage in response to E2. In contrast, the stability of mutant p53 with a missense mutation at the core domain is not affected by E2 treatment, even in ER\(\alpha\)-positive T47-D cells. This cell line expresses a relatively low amount of MDM2. However, the interaction between MDM2 and mutant p53 is not clearly understood yet. Other factors than MDM2 may be implicated in the stability of p53, such as HSP90 and NAD(P)H:quinone oxidoreductase 1 (NQO1). Therefore, further studies are necessary to understand the mechanism of stabilization of mutant p53, which has a longer half-life than that of wild-type p53.

In summary, our findings presented here indicate that wild-type p53 accumulation caused by E2 was due to enhancement of the protein stability. The balance between p60 and p90 forms of MDM2, which was altered by E2, may be important for the MDM2 ubiquitin ligase activity, which is required to degrade p53 protein. Further studies of p60(MDM2) will be required to understand its biological role in p53 regulation and the early stages of breast carcinogenesis.

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