SHORT REPORT

Progesterone inhibition of MDM2 p90 protein in MCF-7 human breast cancer cell line is dependent on p53 levels

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Journal of Molecular and Genetic Medicine (2005), 1(1), 33-37

(Received 11 April 2005; Revised 16 June 2005; Accepted 20 June 2005, Available online 19 August 2005; Published 19 August 2005)

ABSTRACT

The *mdm2* gene encodes several protein isoforms with different molecular weights (p90, p80, p76 and p57). MDM2 p90 (usually considered to be the major MDM2 protein) binds to and inactivates P53. We have recently shown that growth inhibition of MCF-7 human breast cancer cells by progesterone is associated with P53 down-regulation. In this work, we analyzed the expression pattern of MDM2 proteins in three human breast cancer cell lines by western blotting with anti-MDM2 antibodies. We found a prominent expression of MDM2 p57 protein in cell lines which have non-functional P53 protein (T47D and MDA-MB-231) as compared to the p90, p80 isoforms, whereas p90 was the major protein isoform in MCF-7 cells that contain functional P53 protein. When MCF-7 cells were treated with 100 nM of progesterone, MDM2 p90 was inhibited but the highly expressed MDM2 p57 isoform was not. The inhibition of MDM2 p90 protein by progesterone was abrogated in MCF-7 cells transfected with a P53 expressing vector. To our knowledge, this is the first report linking progesterone-induced growth inhibition with down-regulation of the MDM2 protein. We present evidence that reestablishing of P53 expression by transient transfection of P53 cDNA in these cells enhances the expression level of MDM2 p90 isoform. The data indicate that expression of MDM2 p90 is regulated through a P53-dependent pathway in response to progesterone.

KEYWORDS: *MDM2, isoforms, p53, p90, progesterone, MCF-7, breast cancer*

INTRODUCTION

The *mdm2* gene was originally cloned as an amplified gene on a murine double-minute chromosome in the tumorigenic 3T3DM murine cell line (Fakharzadeh et al, 1991). The corresponding human *mdm2* gene was also subsequently identified (Oliner et al, 1992). MDM2 expression is controlled at the transcriptional level from P53-independent (P1) and P53-responsive (P2) promoters (Zauberman et al, 1995), both encoding a 90 kDa full length MDM2 (p90) protein (Brown et al, 1999). In addition, MDM2 proteins of smaller sizes have been identified (Olson et al, 1993; Perry et al, 2000; Bartel et al, 2002). These differently sized proteins arise through either proteolytic cleavage (Pochampally et al, 1998), internal translational initiation (Saucedo et al, 1999) or alternative splicing (Sigalas et al, 1996; Matsumoto, 1998). Although the biochemical functions of these small proteins have not yet been determined, the MDM2-p90 isoform binds to and inactivates P53 tumor suppressor protein suggesting that MDM2 can function as a negative feed-back regulator of P53 (Momand et al, 1992; Barak et al, 1993). Lukas et al (2001) suggested that MDM2 expression is altered in invasive breast cancer and is associated with more aggressive disease. We have recently demonstrated that the progesterone-induced growth inhibition of the MCF-7 human breast cancer cell line was associated with down-regulation of P53 endogenous levels (Alkhalaf and El-Mowafy, 2003). Because the regulation of MDM2 expression by P53 has been proposed by several authors to be the mechanism by which P53 balances its own activity (Juven et al, 1993; Midgley and Lane, 1997; Prives, 1998), we hypothesized that the decrease in P53 levels seen in MCF-7 cells treated with progesterone would affect MDM2 ex-

pression. We report here that in MCF-7 human breast cancer cells treated with progesterone, MDM2 p90 but not MDM2 p57 is down-regulated. To confirm the involvement of P53 in this down-regulation of MDM2, MCF-7 cells were transiently transfected with a P53 expression vector (Alkhalaf and El-Mowafy, 2003). Overexpression of P53 in MCF-7 cells stimulated the MDM2 expression and abrogated the effect of progesterone. The data suggest that expression of MDM2 p90 is regulated via a P53-dependent pathway in MCF-7 human breast cancer cells treated with progesterone.

MATERIALS AND METHODS

Cell lines and culture conditions

The breast cancer cell lines MCF7, T47D, and MDA-MB231 were kindly provided by Bohdan Wasylyk (IGBMC Core Facility, Strasbourg, France). The MCF-7 cells contain functional P53 protein localized at the nucleus (Wasylyk et al, 1999) and classified as progesterone and estrogen receptor positive. The T47D cells have a mutated type of P53 which is localized in the cytoplasm (Schafer et al, 2000) and contain both estrogen and progesterone receptors. The MDA-MB231 cells have non-functional P53 protein (Toillon et al, 2002) and have no functional progesterone and estrogen receptors. The cells were grown in RPMI1640 medium (Gibco BRL) supplemented with 5% fetal bovine serum, glutamine and gentamicin and maintained in a 5% CO_2 humidified atmosphere in a 37 ºC incubator.

Western Blot Analysis

Cells were washed twice with PBS buffer then the pre-heated (95ºC) lysis buffer [20 mM Tris-HCl pH 7.4, 20 mM dithiothreitol (DTT), 2 mM EDTA (sodium salt), 1% (v/v) Triton X-100, 1% (v/v) NP40, 1% (w/v) sodium deoxycholate, 1 mM sodium pyrophosphate, 1 mM sodium orthovandate (prepared in Tris buffer) and 1 mM phenylmethylsulphonyl-fluoride] was added directly to the cell monolayer. The cells were scraped and mixed with a rubber policeman, transferred to Eppendorf tubes and centrifuged at 13000 x g for 5 min. The resulting supernatant was saved and the protein was determined by the Bradford method. Extracts were boiled for 3 min in 2 x SDS buffer. Equal amounts of protein were loaded on 10% (w/v) polyacrylamide gels according to the method of Laemmli and then electrotransferred onto nitro-cellulose membranes. The blots were incubated first with anti-MDM2 (Ab-1, clone IF2) monoclonal antibody (Oncogene Research Product, Calbiochem). The antibody is directed against the N-terminal fragment between amino acid residues 26-169 of human MDM2 (Fakharzadeh et al, 1991) then incubated with peroxidase-conjugated anti-mouse IgG (Jackson Laboratory, USA) at 1/2000 dilution. Immunoreactive bands were visualized by incubation with luminol (according to manufacturer's instructions; ECL Western blotting detection system from Amersham). The blots were stripped and hybridized with P53 (DO1) monoclonal antibodies (Oncogene Research, Cambridge, MA, USA) and processed as for the MDM2 antibodies. The TATA- Binding Protein (TBP) monoclonal antibody was used as a loading control (kindly provided by IGBMC Core Facility, Illkirch, France). Another MDM2 antibody was used, MDM2 (C-18) purchased from Santa Cruz Biotechnology (California, USA). It is an affinity purified rabbit polyclonal antibody raised against a peptide mapping within the carboxyl terminal domain of MDM2 of human origin. A prestained SDS-PAGE molecular weight protein standards (low range) is used to estimate the molecular weights of proteins ((Bio-Rad, UK).

Transient transfection of MCF-7 with P53 cDNA expression vector

MCF-7 cells were maintained in RPMI1640 medium + 5% FBS + antibiotics at 37ºC with 5% (v/v) CO_2 in 36 mm plates (6-well cluster, Nunc, Roskilde, Denmark). They were transfected either with 3 µg CMV expression vector (pcDNA3, obtained from Invitrogen, Paisley, UK) or with 3 µg CMV vector containing human P53 cDNA (kindly provided by the IGBMC core facility, Illkirch, France) by using lipofectamine (Gibco-BRL, Paisley, UK). MCF-7 cells that overexpress P53 as well as the control cells (transfected with the empty vector) were treated with vehicle alone or with 100 nM of progesterone for 24 hr. The total protein extracts were prepared and tested with DO-1, IF2 and TBP monoclonal antibodies as indicated above.

RESULTS

Prominent expression of MDM2 p57 protein in human cancer cell lines

The expression of MDM2 proteins in MCF7, T47D, and MDA-MB231 human breast cancer cell lines was analyzed by Western blotting with anti-MDM2 (Ab-1) monoclonal antibody. Figure 1 shows that, with this antibody, four MDM2 isoforms were detected, the p57, p76, p80, and p90 kDa in all three cell lines. A prominent expression of

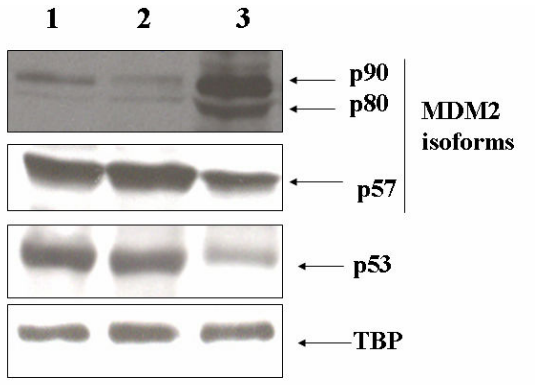

Figure 1. Endogenous levels of P53 and MDM2 isoforms in human breast cancer cell lines. Endogenous levels of MDM2 proteins (p90, p80, p57) and P53 proteins in human breast cancer cell lines MDA-MB231 (lane 1); T47D (lane 2), MCF-7 (lane 3). 100 µg of total protein extract were analyzed by 10% SDS-PAGE, blotted on nitrocellulose membranes and probed with antibodies against MDM2 (IF2), P53 (DO1) and TBP (3G3). Four isoforms of MDM2 were detected and the most abundant is the p57 isoform. P53 protein was detected in all cell lines with different levels. TBP was used as a loading control since it shows the same level in all cell lines.

MDM2 p57 protein as compared to the p90, p80 or p76 was observed in the three cell lines used in this study. MDM2 p90 protein level was dependent on p53 status in the cells. In MCF-7 cells that contained functional P53 protein, the endogenous MDM2 p90 protein level was higher than these in T47D and MDA-MB 230 cell lines (both have mutated P53 proteins). In contrast, MDM2 p57 appeared to be lower in MCF-7 as compared to its levels in the cells which have mutated P53. The high level of MDM2 p57 was observed in other breast cancer cells, a normal breast cell line and in other types of cancer cell lines (data not shown).

Progesterone inhibits MDM2 p90 in MCF-7 human breast cancer cell line

The MCF-7 cell line was used in this experiment because it has high levels of the four MDM2 isoforms and because we had already shown that progesterone down-regulated P53 protein in these cells (Alkhalaf and El-Mowafy, 2003). We investigated whether the P53 inhibition in this cell line would affect the endogenous levels of MDM2 proteins. When MCF-7 cells are treated with 100 nM of progesterone, expression of p90 was specifically inhibited, unlike that of the highly expressed p57 isoform or the TBP protein (Figure 2).

P53 Overexpression stimulates MDM2 expression in MCF-7 human breast cancer cells

The observation that progesterone simultaneously inhibited endogenous MDM2 (Figure 2) and P53 (Alkhalaf and El-Mowafy, 2003) in MCF-7 cells prompted us to check the consequences of re-establishing the P53 expression in the cells by exogenous expression using transient transfection experiments. MCF-7 cells were transfected with expression vector (3 µg/well) for CMV vector and CMV vector containing human P53 cDNA. The cells were treated with vehicle or with 100 nM of progesterone for 24 hr. Western blot analysis was used to estimate the P53 levels achieved by transient transfection. CMV- transfected MCF-7 without progesterone treatment showed normal levels of P53, MDM2 p90 and MDM2 p57. When CMV-transfected MCF-7 cells were treated with 100 nM of progesterone, both MDM2 p90 and P53 were inhibited, unlike the MDM2p57 isoform. MCF-7 cells transfected with CMV+P53 vector showed approximately three fold higher P53 levels than CMV transfected cells (Figure 3) as revealed by densitometry. In these P53 overexpressing MCF-7 cells, the MDM2 p90 level was elevated to reach approximately the same level observed in the CMV transfected MCF7 cells without progesterone treatment. The increase in MDM2 p90 level in MCF-7 cells that overexpress P53 is associated with progesterone treatment suggesting that p53 reestablishing p53 expression enhances the expression of MDM2 p90 and that this increase in MDM2 level is progesterone-dependent.

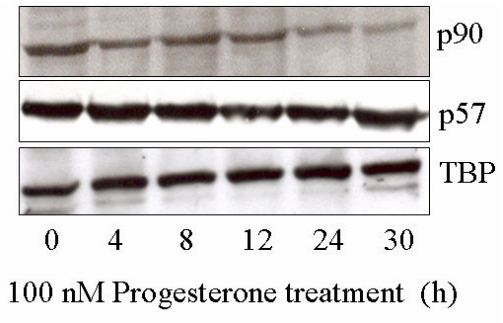

Figure 2. Inhibition of MDM2 p90 protein by progesterone in MCF-7 human breast cancer cells. MCF7 cells were cultured in 5% fetal bovine serum and treated with100 nM of progesterone, harvested at the indicated times. Cellular extracts (100 µg) were analyzed by Western blots for levels of MDM2 (C-18 antibody) and TBP (3G3).

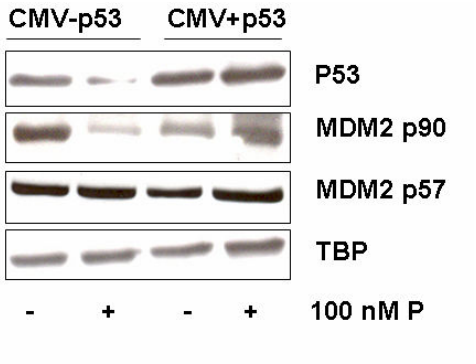

Figure 3. The inhibition of MDM2 p90 protein by progesterone was abrogated in MCF-7 cells transfected with a p53 expressing vector: MCF-7 cells were transfected with expression vector (3 µg/well) for CMV vector and CMV vector containing human P53 cDNA. The cells were treated with vehicle or with 100 nM of progesterone (P) for 24 hours. 20 µg of extracts were subjected to Western blotting analysis to determine the relative amounts of P53, MDM2 p90, MDM2 p57 and TBP (used as a loading control) proteins. MCF-7 cells transfected with CMV-P53 vector contained over three fold of P53 than CMV transfected cells. The cells showed increase in MDM2 p90 but not in MDM2 p57 protein levels. The increase in the MDM2 p90 in response to P53 overexpression has the same magnitude observed in cells not treated with progesterone.

DISCUSSION

More than 40 different splice variants of MDM2 transcripts have been identified both in tumors and normal tissues (Gudas et al, 1995; Lukas et al, 2001; Bartel et al, 2002; Phelps et al, 2003). At the protein level at least five MDM2 proteins (p90, p80, p74/76, p70, p57/60) have been described (Oslon et al, 1993; Haider et al, 1997; Pochampally et al, 1998; Pochampally et al, 1999; Saucedo et al, 1999; Bartl et al, 2003). It was demonstrated that these differently-sized MDM2 isoforms may arise through either proteolytic cleavage (Pochampally et al, 1998), internal translational initiation (Saucedo et al, 1999) or alternative splicing (Sigalas et al, 1996; Matsumoto et al, 1998). Although the biochemical functions of these proteins have not yet been determined, their exis-

tence suggests that MDM2 may be involved in additional unknown functions.

The expression of MDM2 is known to be induced by P53 as Landers et al. 1997 demonstrated that the high level of mdm2 overexpression in human tumor cells containing elevated levels of wild-type P53 resulted from enhanced translation at the p53-responsive promoter region of the mdm2 gene (Landers et al, 1997). Recent evidence has shown that MDM2 expression can also be regulated via P53-independent pathways (Phelps et al, 2003). Our data show that in cells with functional P53 proteins, endogenous MDM2 p90 was the major MDM2 protein isoform and that cells with a mutated P53 gene showed very low levels of the MDM2 p90 protein. The present data show also that progesterone down-regulates the endogenous level of MDM2 p90. This down-regulation of MDM2 p90 may be associated with down-regulation of the endogenous P53 level observed in the same conditions (Alkhalaf and El-Mowafy, 2003). By contrast, re-establishing the P53 endogenous expression in MCF-7 by transient expression led to elevation of MDM2 p90 indicating the possible involvement of the P53 pathway in the regulation of MDM2 p90.

Overexpression of the MDM2 proteins is often observed in breast cancer tissues and cell lines (Sheikh et al, 1993; McCann et al, 1995; Pinkas et al, 1999). Bueso-Ramos et al (Bueso-Ramos et al, 1996) suggested that MDM-2 p57 protein represents the main MDM-2 protein altered in breast carcinomas: they found MDM-2 overexpression in 15 of 21 breast carcinoma tissue samples. Ten of these fifteen cases overexpressed MDM2 p57 protein, while two cases overexpressed both MDM2 p57 and MDM2 p90, and three cases overexpressed only MDM2 p90. However, the status of the P53 gene in the tumors overexpressing the MDM2 p57 was not reported. Our data show that MDM2 p57 is the predominant isoform in cancer cells that have mutations in the P53 gene. However, further studies are required to fully understand the correlation between the expression of the different forms of MDM2 proteins and the P53 status in breast cancer tumors.

ACKNOWLEDGMENTS

We thank Professor Christopher H. J. Ford for his critical reading of the manuscript and Maya Bakir and Lilly Verghese for technical assistance. This work was supported by grant MB 04/04 awarded by Kuwait University Research Administration.

STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

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