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p53-independent upregulation of $p21^{WAF1}$ in NIH 3T3 cells malignantly transformed by mot-2

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ABSTRACT

Mot-2 protein is shown to interact with p53 and inhibit its transcriptional activation function. Mot-2 overexpressing stable clones of NIH 3T3 cells were malignantly transformed, however, they had a high level of expression of a p53 downstream gene, $p21^{WAF1}$. The present study was undertaken to elucidate possible molecular mechanism(s) of such upregulation. An increased level of $p21^{WAF1}$ expression was detected in stable transfectants although an exogenous reporter gene driven by $p21^{WAF1}$ promoter exhibited lower activity in these cells suggesting that some post-transcriptional mechanism contributes to upregulation. Western analyses of transient and stable clones revealed that upregulation of $p21^{WAF1}$ in stable NIH 3T3/mot-2 cells may be mediated by cyclin D1 and cdk-2.

Key words: *Mouse fibroblasts, malignant transformation, mot-2, p53, $p21^{WAF1}$, $p16^{INK4a}$, cyclin D1, cdk-2*

INTRODUCTION

Multiple cellular functions of p53, a most frequently mutated tumor suppressor in human cancers, are involved in its ability to induce and regulate cell cycle arrest and apoptosis[1,2]. Among these are its ability to bind to specific DNA sequences and activate transcription of genes such as, mdm-2, $p21^{WAF1}$, gadd45, bax and IGFBP-3[3-6]. $p21^{WAF1}$, the pioneer member of p21 family of cyclin-cdk inhibitor class of proteins ($p21^{WAF1}$, $p27^{Kip1}$ and $p57^{Kip2}$), that bind to cyclin D1[7,8] has been implicated as a growth arrest mediator in p53-tumour suppression, cellular senescence and terminal differentiation. Similarly, another class of cyclin-cdk inhibitors, INK4 family members ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) that bind to cdk-4 and cdk-6[9-11] regulate cell cycle progression. By inactivating the cyclin-cdk complexes, these inhibitors block phosphorylation of RB family of proteins (RB) and abrogate release of E2F (required for cell cycle progression) from RB-E2F complexes[12]. Number of studies have supported that $p21^{WAF1}$ functions during normal and induced cellular senescence[13-17]. However, mice lacking $p21^{WAF1}$ have normal phenotype[18], [19] and its upregulation has been detected by mitogenic and growth stimulatory signals[20],[21]. These data suggested that physiological function of $p21^{WAF1}$ may not be limited to execution of cell cycle arrest program.

Mot-2, a member of hsp70 family protein, was seen to interact with p53 and inactivate its transactivation function by abrogating its nuclear translocation [22]. While characterizing mouse fibroblasts stably transfected with mot-2, we found that in spite of inactivation of the p53 function by mot-2, these cells have high level of $p21^{WAF1}$. Studies were therefore undertaken to elucidate the

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mechanism(s) of such upregulation of p21^{WAF1}. Consistent with the protein levels, Northern analysis revealed an upregulation of p21^{WAF1}. A comparative analysis of p21^{WAF1}, cyclin D1 and cdk-2 in stably- and transiently- transfected cells suggested that the upregulation of p21WAF1 in stable NIH 3T3/mot-2 cells may occur possibly by post-transcriptional and p53-independent mechanism(s) such as those mediated by cyclin D1, cdk-2 and E2F.

MATERIAL AND METHODS

Cell culture

NIH 3T3 cells were transfected (LipofectAMINE^{TM}, Life Technologies, Inc) with expression plasmid encoding mouse mot-2 protein and the stable clones were isolated and maintained in 50 μg/ml G418-supplemented growth medium (Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as described[23]. Normal mouse embryonic fibroblasts (CMEF) from CD1-ICR mouse and their spontaneously immortalized clone, RS-4, were serially passaged similarly by 1:8 subculturing in DMEM.

Northern blot analysis

RNA from NIH 3T3 and its mot-2 derivatives were extracted by using Isogen (GibcoBRL). Northern analysis for p21^{WAF1} was performed by using 10 mg of total RNA, separated on 1.5% denaturing agarose gel, transferred to Hybond N (Amersham) membrane by capillary transfer, and probed with the 32P-radiolabeled mouse p21^{WAF1} cDNA (kindly provided by Dr. Olivia M. Pereira-Smith).

Western blot analysis

The protein sample (10-20 mg) separated on an SDS-polyacrylamide gel was electroblotted onto a nitrocellulose membrane (BA85, Schleicher and Schuell) using a semidry transfer blotter (Biometra, Tokyo). Immunoassays were performed with anti-mortalin[24], -p21 (M-19, Santa Cruz), -p16^{INK4a} (M156, Santa Cruz), -cdk-2 (M2, Santa Cruz), -cdk-4 (C-22, Santa Cruz), -cdk-6 (c-21, Santa Cruz), -cyclin D1 (72-13G, Santa Cruz) or anti-actin (Boehringer Mannheim) antibodies. The immunocomplexes formed were visualized either with horseradish peroxidase (HRP) or alkaline phosphate-conjugated anti-mouse/rabbit immunoglobulin G (IgG) (ECL kit, Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

NIH 3T3/mot-2 cells show an upregulation of p21WAF1 expression

Western analysis with anti-p21 antibody revealed a high expression level of p21^{WAF1} in NIH 3T3/mot-2 cells as compared to untransfected and vector-transfected cells. This was contrary to the inactivation of p53 function by mot-2[22],[25]. We first ascertained that the band detected on Western blot was indeed p21^{WAF1} by using serially passaged normal mouse embryonic fibroblasts (CMEF) and NIH 3T3 cells cultured at different density (Fig 1A). In agreement to known pattern of p21WAF1 expression in senescence human cells [13],[26], it was seen to undergo upregulation with in vitro aging of mouse cells. CMEF cells at passage 6-8 showed senescent morphology following which spontaneously immortalized clones emerged. One of such clones, RS-4 that showed mutant p53 [27] was also assayed for p21 expression level (Fig 1A). In spite of the presence of mutant p53, RS-4 cells showed the p21^{WAF1} expression, albiet at level less than the one detected in cells at senescent phase (passage 8), suggesting p53-independent expression of p21WAF1. However, p21^{WAF1} expression in RS-4 cells was not induced by high-density culture in contrast to NIH 3T3 cells that have wild type p53 protein (Fig 1A). These data established that the band detected on our Westerns represented p21^{WAF1}. NIH 3T3/mot-2 cells (two independent clones) showed a high level of expression of p21^{WAF1} even at low-density culture and did not support inactivation of p53 function by mot-2 in these cells (Fig 1A). A p53-independent increase in the level of p21^{WAF1} expression of by post-transcriptional[28] and post-translational[29] mechanism has been reported. Therefore, to detect the level at which p21^{WAF1} had undergone upregulation in NIH 3T3/mot-2 cells, we next performed Northern analysis. Consistent with Western blot analysis, a high level of transcript was apparent in NIH 3T3/mot-2 cells (Fig 1B) implementing that in these cells upregulation of p21^{WAF1} occurred at transcriptional level. Since these cells show inactivation of p53 function[22],[25], it is likely that upregulation of p21^{WAF1} occurred either by p53-independent pathway or involves post-transcriptional mechanisms. We also performed p53 dependent reporter assays in NIH 3T3 and NIH 3T3/mot-2 cells. The activity of the full length p21^{WAF1} promoter reporter plasmid was three-five fold high in NIH 3T3 cells in different experiments[22],[25] and data not shown]. This suggested that the high level of p21^{WAF1} transcript in NIH 3T3/mot-2 cells is either regulated by elements located further upstream to the 2501 bp in the promoter used in the reporter plasmid or by post-transcriptional mechanism(s).

Identity of the parent NIH 3T3 and its mot-2 derivative was affirmed by Western blot analysis with anti-p16^{INK4a} antibody (Fig 1C). Consistent with the upregulation of p16^{INK4A} during senescence of mouse and human cells[9], [30-33] we detected its high level of expression in late-passage mouse cells. And, consistent with the biallelic deletion of INK4a locus in NIH 3T3 cells[34] p16^{INK4a} was also not detected in either NIH 3T3 or NIH 3T3/mot-2 cells by Western blot analysis; confirming the identity of mot-2 derivatives to parent NIH 3T3 cells. RS-4 cells on the other hand have p16^{INK4a}

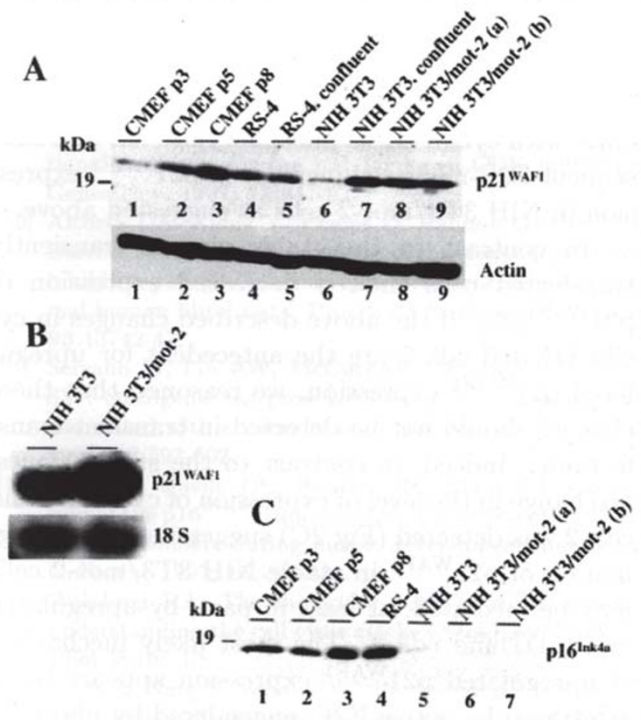

Fig 1. **A.** Western blot analysis with anti-p21^{WAF1} antibody in celllysates from normal (CMEF) cells at different passages (p), p3-p8 and immortal cells (RS-4, NIH 3T3 and NIH 3T3/mot-2) at different densities as indicated. A high level of p21WAF1 expression was detected in normal cells at later passages (senescence), in high density culture of NIH 3T3 cells and in low density culture of NIH 3T3/ mot-2 cells. Lower panel shows equal amount of protein loading as detected by probing of the same membrane with anti-actin antibody. **B.** Northern analysis for p21^{WAF1}. A high level of p21^{WAF1} transcript was detected in NIH 3T3/mot-2 cells. The membrane was hybridized with 18S oligonucleotide probe for loading control. **C.** Western blot analysis of indicated cells with anti-p16^{INK4a} antibody. An increase in p16^{INK4a} was detected in CMEF during serial passaging. Similar to NIH 3T3, NIH 3T3/mot-2 cells lack the expression of p16 ^{INK4a} and served as a fingerprint for these cells.

expression and were nonmalignant in their growth properties. It has been shown that mice that carry targeted deletions of p16^{INK4a} locus developed spontaneous tumors at an early age and are highly sensitive to carcinogenic treatment[35]. p16-/- mouse embryonic fibroblasts proliferate rapidly in culture, grow at high densities and show a high colony forming efficiency. An introduction of p16^{INK4a} to human gliomas that undergo frequent loss of p16^{INK4a} induces cellular senescence[32]. Such functions of p16^{INK4a} and its presence in RS-4 cells may be related to their nonmalignant phenotype in spite of the presence of mutant p53 and other transformation favoring characteristics[27], [36].

Taken together, these data presented evidence for post-transcriptional and p53-independent upregulation of p21^{WAF1} expression. Other examples of p53-independent increase in p21^{WAF1} expression include hepatocytes following carbon tetrachloride intoxication[37], hematopoietic and hepatoma cells during differentiation[38], human melanoma cells during IFN-b and MEZ induced growth arrest and terminal differentiation[39], myeloid leukemic cells during terminal differentiation by a hormonal form of vitamin D[40].

An amplification of p21WAF1 from NIH 3T3 and NIH 3T3/mot-2 cells by RT-PCR and sequence analysis (data not shown) of the amplified DNA product confirmed the wild type nature of p21^{WAF1} in malignantly transformed NIH 3T3/mot-2 cells. This indicated that an increased level of expression of p21^{WAF1} does not correlate with its cell cycle arrest function and may have some other function (s). In this context, elevated levels of p21^{WAF1} expression have been detected in gliomas[41], non small cell lung carcinomas[21],[42], invasive cervical cancer[43] and bladder carcinomas[44]. A 50-100 fold increase in p21^{WAF1} expression has been detected in NIH 3T3 cells transformed with activated Ras oncogene[20].

Cyclin D1 and cdk-2 are upregulated in stable NIH 3T3/mot-2 cells

An ectopic expression of cyclin D1 has been found to result in increased expression of p21^{WAF1} in human glioma and rodent fibroblasts that were morphologically transformed[45]. Such induction of p21^{WAF1} has been suggested to be mediated by

E2F; an E2F binding site is found in p21Waf1 promoter[46]. A positive feed back regulation between cyclin D1 and p21^{WAF1} has been proposed. Cyclin D1/cdk-4 phosphorylate pRb and generate free E2F that can initiate p21^{WAF1} transcription. Under the conditions when cyclin D1 levels are higher than p21^{WAF1}, cells continue to divide and are unlikely to respond to p21-mediated growth inhibitory signals resulting into cellular transformation. Conversely, when p21^{WAF1} levels are greater than cyclin D1, cell cycle machinery is suppressed and/or shifted towards the direction of differentiation. Since NIH 3T3/mot-2 cells were seen to have a higher level of expression of p21^{WAF1} as compared to NIH 3T3 and CMEF cells, we next analyzed the expression of cyclin D1 in these cells (Fig 2A). A higher level of cyclin D1 was detected in NIH 3T3/mot-2 cells elucidating a factor that may positively affect p21^{WAF1} expression by abrogation of RB function through phosphorylation.

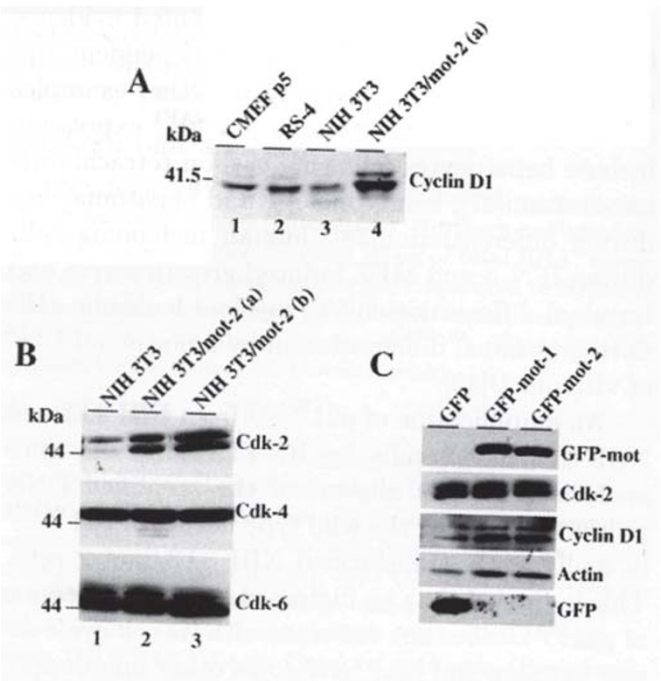

Fig 2. A. Western blot analysis for cyclin D1 in normal (CMEF), immortal (RS-4 and NIH 3T3) and malignantly transformed (NIH 3T3/mot-2) cells. An increase in expression level of cyclin D1 was detected in NIH 3T3/mot-2 cells. **B.** Western blot analysis of NIH 3T3 and its mot-2 derivative with anti-cdk-2, -4 and -6 antibodies. An increase in expression was detected for cdk-2 in mot-2 derivative. **C.** Western blot analysis of NIH 3T3 cells transiently transfected with GFP-tagged mot-1 and mot-2 proteins with anti-GFP, -cdk-2, -cyclin D1 and -actin antibodies. An equal level of expression was detected in control and transiently transfected cells.

Since active complexes of cyclin-cdks are essential for phosphorylation/ablation of tumor suppressor function of pRb for the release free E2F, we next analyzed expression profiles for cdk-2, cdk-4 and cdk-6 in NIH 3T3/mot-2 cells (Fig 2B). A low molecular mass band (the active form) of cdk-2 was detected at higher level in NIH 3T3/mot-2 as compared to NIH 3T3 cells. These data submit that such upregulated cdk-2 expression is likely to cooperate with cyclin D1 to phosphorylate RB and subsequent E2F-mediated increase in p21^{WAF1} expression in NIH 3T3/mot-2 cells as suggested above.

In contrast to the stable clones, transiently transfected cells showed decreased expression of p21^{WAF1} [22]. If the above described changes in cyclin D1 and cdk-2 are the antecedent for upregulated p21^{WAF1} expression, we reasoned that these changes should not be detected in transient transfectants. Indeed, in contrast to the stable clones, no change in the level of expression of cyclin D1 and cdk-2 was detected (Fig 2C) suggesting that upregulation of p21^{WAF1} in stable NIH 3T3/mot-2 cells may be mediated, at least in part, by upregulated cyclin D1 and cdk-2. The most likely mechanism of upregulated p21^{WAF1} expression appears to be arbitrated by active E2F, engendered by phosphorylation of RB that is caused by upregulated cyclin D1 and cdk-2. Mechanism of mot-2 interposed active cyclin D1 and cdk-2 levels in stable NIH 3T3/mot-2 cells and its absence in cells transiently transfected with plasmids encoding GFP-tagged mot-2 protein warrant further investigations.

In summary we have shown p53-independent upregulation of p21^{WAF1} in NIH 3T3 cells that are malignantly transformed by mot-2.

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