Single chain antibody against the common epitope of mutant p53 restores wild-type activity to mutant p53 protein

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Abstract Here, we describe the biological activity of ME1, a mouse single chain Fv fragment (scFv) against the common epitope of mutant p53, which is efficiently expressed in mammalian cells. We found that in vivo interaction of the conformational p53 mutant R175H protein with the scFv resulted in the acquisition of wild-type p53 characteristics, manifested in trans-activation of p21, as well as induction of apoptosis. Moreover, antibody binding leads to abrogation of the mutant p53 mediated “gain of function” as estimated by downregulation of EGR-1, a transcriptional target of mutant p53. These findings suggest that the scFv restores wild-type properties to mutant p53.

Keywords: p53; p53-R175H mutant; Single chain Fv fragment; Activity restoration

1. Introduction

Mutations in the p53 tumor suppressor gene are the most common genetic alterations and occur in more than half of all human tumors [1]. Most of these alterations are missense mutations in the DNA-binding core domain responsible for sequence-specific binding of wild-type p53 protein to target genes.

Involvement of p53 mutants in cancer progression was suggested to be associated with either trans-dominant suppression of wild-type p53 or a wild-type p53-independent oncogenic “gain of function” [2]. This gain of function involves the ability of mutant p53 to transactivate or repress specific genes, different from those affected by wild-type p53, that mediate the various oncogenic activities of these mutants.

Given the active role of p53 mutants in promoting tumorigenicity, efforts are being made to inactivate their function, or restore wild-type activity employing various strategies reviewed in [2,3]. These include the introduction of second site suppressor mutations that can at least partially restore specific DNA binding to mutant p53 [4,5], synthetic peptides derived from the C-terminus of the p53 protein [6–8], or the CDB3, a p53-binding protein (p53BP2) derived compound [9], and low molecular weight compounds, such as CP-31398 [10] and PRIMA-1 [11].

The “chaperone like” activity of antibodies, which was shown to modulate protein conformation [12], as well as the intracellular localization of p53, prompted us to use intracellular expression of specific recombinant site-directed antibodies as a tool for restoring wild-type p53 activity.

More than 90% of the mutations in p53 produce a specific conformational change which results in exposure of an epitope, FRHHSV that is otherwise hidden in the hydrophobic core of the molecule. Previously, we have reported the generation of a scFv termed ME1 against this epitope [13]. In this study, we describe the biological activity of the ME1 antibody. We demonstrate that intracellularly expressed scFv ME1 leads to restoration of trans-activation activity, enhancement of apoptosis and abrogation of the “gain of function”. These findings suggest the importance of such antibodies in the treatment of tumors expressing mutant p53.

2. Materials and methods

2.1. Cell lines, plasmids and transfections

Cell line H1299, derived from a human large cell lung carcinoma was obtained from ATCC. H1299-R175H was derived by stable transfection of H1299 cells with the pcDNA3-based expression vector constructed by G. Blandino carrying the p53-R175H core domain mutant obtained from B. Vogelstein. SKBR3, derived from a human breast carcinoma, endogenously expressing the p53 mutation R175H was obtained from I. Benhar. The reporter construct RGC-Luciferase was used for Luciferase trans-activation experiments. Both cell lines were maintained in RPMI (Sigma) medium supplemented with 10% FCS (Sigma). pc53-SN3 plasmid expressing the wild-type p53 protein was obtained from B. Vogelstein.

The plasmid pMalC-NN [14] was obtained from I. Benhar. The pShooter plasmids pCMV/myc/cyto and pCMV/myc/nuc were from Invitrogen. A plasmid carrying β-galactosidase was co-transfected in all experiments and used for normalization of transfection efficiency. In all experiments, Fugene 6 (Roche Diagnostics) was used as a transfection reagent, according to the manufacturers’ recommendations.

2.2. MEI scFv expression and purification

Escherichia coli cells, transformed with pMalC-NN carrying scFv ME1, were grown in LB medium supplemented with 100 μg/ml ampicillin and 1% (w/v) glucose. When the culture reached A600 of 0.6–0.9, it was induced with 0.5 mM IPTG at 30 ºC for four hours. Cell extracts were prepared in 20 mM Tris–HCl (pH 7.4), 1 mM EDTA and 200 mM NaCl by sonication. The extracts were clarified by centrifugation at 20000 × g. MBP-scFv fusions were purified on an amylose resin column (New England Biolabs, USA).

2.3. Western blot analysis

Cells were lysed in TBL buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholate and 0.1% SDS) supplemented with EDTA free protease inhibitor (Roche). Protein concentrations were determined using the BCA protein assay kit (Pierce) according to the manufacturers’ instructions. Equivalent amounts of

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protein were resolved on SDS-polyacrylamide gels, electrophoresed on 0.45 Immobilon-P membranes (Millipore), and incubated for 1 h at room temperature with monoclonal anti-MBP diluted 1:5000 (Sigma, USA), or rabbit polyclonal anti-EGR-1 1:200 dilution (Santa Cruz Biotechnology, Inc.), or rabbit polyclonal anti-p21 1:250 dilution (Santa Cruz Biotechnology, Inc.) or anti-tubulin 1:1000 (Sigma). All dilutions were in 1% w/v low fat milk. Blots were then incubated with HRP-conjugated anti-mouse or anti-rabbit IgG 1:5000, for 1 h and visualized using the ECL kit (Amersham) according to the manufacturers’ instructions.

2.4. Luciferase assays
H1299 cells (4 x 10^5/well) were transfected with 100 ng of a reporter plasmid, RGC-Luciferase, in combination with different amounts of plasmids encoding MBP-ME1-cyto and MBP-ME1-nuc. 1 ng of pcDNA3-human p53-R175H and 100 ng of CMV-β-galactosidase. Luciferase activity was assayed 48 h post-transfection using the Luciferase reporter assay system (Promega) according to the manufacturers’ instructions. Transfections were done in triplicates and normalized to β-galactosidase activity as an internal transfection control. Luminescence was determined with the Rosys-Anthos Lucy 3 luminometer.

2.5. β-Galactosidase enzyme assay
A sample of 5 μl of cell lysate was mixed with 200 μl of LacZ buffer (60 mM Na2CO3, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4) and 20 μl of substrate (2 mg/ml ONPG in Na2CO3, 40 mM NaH2PO4). The reaction was incubated at 37 °C until color was developed and absorbance was determined at 0.D.420.

2.6. ELISA
Recombinant human wild-type p53 or core domain mutants p53-R175H, p53-R248W and p53-R273H, expressed in insect cells, were absorbed to 96-well PVC assay plates (Falcon) overnight at 4 °C. Purified bacterial soluble MBP-ME1 scFv was used. For competition assay, the plates were coated with streptavidin overnight at 4 °C, washed and then incubated with biotin-FRHSVV for 1 h. The purified MBP-ME1 was pre-incubated with FRHSVV peptide in solution for 1 h and then incubated with biotin-FRHSVV for 1 h. The purified MBP-ME1 scFv antibody directed against the common epitope of mutant p53, termed ME1 [13], was impaired by its instability. As scFvs can be stabilized by fusing to MBP [14], we cloned the ME1 scFv sequence into the pMalC-NN plasmid. Upon induction with IPTG the ME1 scFv fused to MBP accumulated to high levels in the cells (Fig. 1A, lane 2). A large amount of the fusion protein was found in the soluble fraction (Fig. 1A, lane 3) which was purified to near homogeninity on an amyllose resin (Fig. 1A, lane 4).

Following purification, immunological binding activity of the MBP-ME1 scFv was tested in a direct and a competitive ELISA. The wells were coated with the peptide FRHSVV and the binding capacity of purified fusion MBP-scFv prior and following incubation with various amounts of the soluble FRHSVV peptide was measured. As shown in Fig. 1B, the fusion protein MBP-ME1 scFv retained binding properties to the common epitope of mutant p53. Pre-incubation of the ME1 antibody with soluble peptide FRHSVV prior testing resulted in a dose-dependent inhibition of binding to the ELISA plate.

We used recombinant wild-type p53 as well as three recombinant p53 mutant proteins p53-R175H, p53-R248W and p53-R273H, produced in insect cells, in an ELISA assay to test whether ME1 scFv binds to the epitope when presented in the context of a whole mutant p53 molecule. Indeed, the antibody was able to bind all three mutant proteins but not the wild-type p53 protein (data not shown).

3.2. Intracellular expression of the ME1 scFv gene in mammalian cells
Biological activity of the ME1 antibody was measured using two expression vectors. The scFv ME1 was subcloned into the NeoI–NotI sites of the vectors pCMV/myc/cyto and pCMV/myc/nuc, which utilize the CMV promoter to derive expression of the scFv in the cytoplasm or in the nucleus, respectively. The ability of the scFv ME1 to be expressed in mammalian cells was determined by transient transfection of p53−/− H1299 human lung carcinoma cell line with 10 μg of each of these two expression vectors. Intracellular expression of ME1 fused to MBP was determined by immunofluorescence staining with anti-MBP. Abundant accumulation of the fusion antibody was observed in the cytoplasm when cells were transfected with the ME1-cyto and in the nucleus when transfected with ME1-nuc (Fig. 2A). A band corresponding to the ME1 fusion protein (~65 kDa) was detected in a Western blot of transfected cell lysates using anti-MBP antibody (Fig. 2B).

These results indicate that ME1 scFv fused to MBP can be expressed both in the cytoplasm and nucleus of mammalian cells. Furthermore, the expressed fusion scFv proteins are stable and do not undergo rapid degradation, as previously observed with the non-fused antibody [13].

3.3. scFv ME1 facilitates transcriptional activity of mutant p53-R175H
Wild-type p53 is a transcription factor that was shown to induce expression of target genes through binding to a specific DNA responsive element [15]. To examine whether interaction of mutant p53 with scFv ME1 may restore transcriptional activity to mutant p53, we used the RGC-Luciferase reporter construct containing several wild-type p53 responsive elements upstream to the firefly
Luciferase gene. For this purpose, we used H1299 R175H cells stably expressing the p53-R175H mutant (Fig. 3 A) or H1299 cells transiently expressing R175H (Fig. 3 B). Co-transfection of both cell lines with 100 ng RGC-Luciferase in combination with different amounts of ME1-cyto or ME1-nuc resulted in an increase in Luciferase activity. When cells were transfected with anti-β-galactosidase, an irrelevant MBP-scFv plasmid expressed in the cytoplasm, no effect on Luciferase activity was observed, indicating that binding of mutant p53 R175H to the wild-type p53 responsive element following ME1 scFvs expression is specific. When cells were co-transfected with wild-type p53 in combination with the ME1 scFv no difference in Luciferase activity was observed compared to levels obtained when cells were transfected with wild-type p53 alone, confirming the specificity of the ME1 scFv to mutant p53 (data not shown).

To further substantiate the above observation, we have examined changes in p21 expression, a “bona fida” wild-type p53 target gene. To that end, we transfected H1299-R175H cells, stably expressing mutant p53, with ME1-cyto or ME1-nuc. Western blot analysis of cell extracts probed with anti-p21 revealed an elevation in the level of p21 protein when ME1 scFv was expressed in the nucleus, compared to cells transfected with an empty plasmid. No change was observed when the antibody was expressed in the cytoplasm (Fig. 3 C). These results support the conclusion that mutant p53 gained a wild-type p53 biological activity at least as estimated by these assays.
3.4. ME1 scFv abrogates the gain of function activity of mutant p53

Mutant p53 contributes to the malignant process by acquisition of novel functions [16]. Transactivation of specific target genes that are not activated by wild-type p53 is one such function. Recently, we found that mutant p53 trans-activates the Early Growth Response 1 (EGR-1) gene [17]. We examined whether interaction of mutant p53 with ME1 scFv modifies this mutant p53-mediated activity. We found that cells stably expressing mutant p53-R175H when transfected with the scFv ME1 construct, showed a moderate decrease in ERG-1 protein level when the antibody was expressed in the cytoplasm, whereas a dramatic decrease was observed when expressed in the nucleus (Fig. 3D). This reinforces that exposure of mutant p53 to ME1 scFv induces changes in the mutant p53 molecule.

3.5. Interaction of mutant p53 with ME1 scFvs induces apoptosis

The apoptotic activity of p53 is a hallmark of this tumor suppressor gene [18]. Apoptosis is a multistep process which includes the activation of a cascade of intracellular cysteine proteases, designated caspases [19]. We used caspase 3/7 activity as a measure for apoptosis. H1299 cells were co-transfected with 1 ng p53R175H plasmid in combination with various amounts of ME1 scFvs. Caspase 3/7 activity was determined 48 h post-transfection. Both ME1 scFv constructs, expressed in the cytoplasm or in the nucleus, induced a dose-dependent significant increase in caspase 3/7 activity (Fig. 4). Expression of anti-β-galactosidase scFv in the cytoplasm, a non-relevant scFv, did not culminate in induction of caspase activity. The amount of ME1 scFv required for activation of caspase activity was higher when expressed in the cytoplasm than the amount needed when expressed in the nucleus. The results obtained in this assay confirmed that mutant p53 seem to lose a mutant specific feature upon exposure to ME1 scFv, and thus exhibit reduced apoptotic blocking activity. To further strengthen these results, we used FACS analysis to determine the cell cycle profile and degree of apoptosis upon expression of ME1 in H1299 p53−/−, H1299 stably expressing R175H as well as SKBR3, a tumor cell line endogenously expressing mutant p53-R175H. Expression of ME1 in the nucleus of either H1299 R175H or SKBR3 cells resulted in enhanced apoptosis in a dose-dependent manner. The cells were transfected with 1, 1.7 and 3.3 μg of ME1-nuc which caused an induction of apoptosis of 17%, 26% and 30%, respectively, in H1299 R175H cells, and 14%, 23% and 32% in SKBR3 cells. The level of apoptotic cells was unaffected in H1299 p53−/− cells. No effect was observed when the cells were transfected with 3.3 μg of a non-relevant β-Gal antibody, or when ME1 was expressed in the cytoplasm (Fig. 5).

4. Discussion

Many of the p53 core domain mutants assume a common conformational structural change [20]. The extent of the conformational change varies in different mutants according to the nature of the missense mutation, resulting in the exposure of the mutant p53 common epitope FRH5SVV to different extents [21]. Taking these findings into consideration, we have generated a single chain antibody [13] which specifically binds to the mutant p53 common epitope when displayed on the whole p53 mutant molecules. Mutant p53-R175H is known to be the most severe conformational p53 mutant causing a large destabilization effect of the mutant protein, mutation R248W causes an intermediate destabilization effect, while the p53-R273H molecule has almost a wild-type conformation. The binding capacity of scFv ME1 to these three p53 mutants was found to be in accordance with the extent of their conformational change, where the highest binding was found with p53-R175H and the lowest binding with p53-R273H.
Exposure of mutant p53-R175H to the scFv expressed, either in the cytoplasm or in the nucleus, induced binding of mutant p53 to wild-type p53 responsive elements, which in turn caused **trans**-activation of specific downstream p53 target genes. Higher amounts of ME1 were required in the cytoplasm as compared to those in the nucleus for **trans**-activation. This can be attributed to the fact that the antibodies exhibit different stabilities and protein folding at various sub-cellular compartments. Another explanation may be associated with the efficiency of antibody translocation from the cytoplasm to the nucleus. When expressed in the cytoplasm, it is most likely that the scFv/p53 complex has to reach the nucleus, where p53 is known to exert its activity. Translocation of the scFv/p53 complex to the nucleus may be inefficient, while when expressed in the nucleus it is not dependent upon p53 trafficking. The differences in the degree of transcription activation observed in cells stably expressing the mutant p53 protein as compared to those that were transiently transfected may reflect the difference in the expression levels as well as the distribution of mutant p53 protein in these cells.

The “chaperone like” activity of antibodies is well documented, reviewed in [13], they have been shown to change folding and unfolding states of proteins upon binding. We assume that the binding of the scFv ME1 to the p53 mutant protein rendered the mutant molecule thermodynamically more stable driving it from an unfolded state to a more favorable one. This

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**Fig. 4.** Expression of ME1 scFv enhances caspase 3/7 activity. H1299 cells were transiently transfected with 1 ng of pcDNA3-R175H plasmid without or in combination with various amounts of ME1-cyto (checkered), ME1-nuc (striped), as indicated, 1 ng of pc3-SN3 (black) or 50 ng of anti β-galactosidase scFv (white) and Caspase activity was determined. Caspase activity in cells transfected with pcDNA3-R175H alone were subtracted from caspase activity values obtained in each experimental group.

**Fig. 5.** Expression of ME1 induces apoptosis in cells expressing mutant p53 Cell cycle distribution of H1299 cells, H1299 stably expressing mutant p53-R175H and SKBR3 cells transfected with 3.3 μg ME1-cyto, 3.3 μg anti β-galactosidase scFv or various amounts of ME1-nuc, as indicated. Numbers indicate the percentage of apoptotic cells.
increased stability is accompanied by the gain of activities typical to the wild-type p53 protein. It is now established that the molecular chaperones HSP70, HSP40 and HSP90 form a complex that can refold denatured proteins as well as protect proteins from unfolding, but they have been also shown to prevent drug or radiation-dependent apoptosis in cells. Stable interactions were observed between these HSPs and mutant p53 protein in tumor cells, but not with native wild-type p53. The binding site for HSPs within the p53 protein was mapped to the hydrophobic pocket of the core domain, which is exposed in the unfolded p53 mutant protein but berried in the wild-type p53 molecule [22]. The epitope that is recognized by scFv ME1 resides in the same hydrophobic pocket and like the HSPs binds exclusively to mutant p53. Depletion of Hsp70 selectively kills cancer cells not only in cell culture but also in various tumor xenografts in mice [23]. Recently, it was shown that depletion of Hsp70-2, a member of the Hsp70 family, resulted in upregulation of p21 and MIC-1. The expression of both genes is rapidly induced by a variety of cellular stresses in a p53-dependent manner [24]. It is tempting to speculate that binding of scFv ME1 to mutant p53 excludes the binding of the HSPs to p53, or that scFv ME1 having a high affinity binding constant may displace the HSPs complex and by this releasing the inhibition of apoptosis conferred by the HSPs.

Apoptosis is a hallmark for wild-type p53 activity. The link between p53, caspase activation and apoptosis has been demonstrated [25,26]. We found that co-expression of mutant p53 with scFv ME1 caused an increase in caspase 3/7 activity, indicating that binding of the antibody to mutant p53 may result in transactivation of transcriptional targets leading to apoptosis. Induction of apoptosis upon expression of scFv ME1 in the nucleus was demonstrated in cells transfected with the p53-R175H mutant as well as in a cell line that expresses this mutation endogenously. Moreover, interaction of mutant p53 with scFv ME1 caused increased binding to the wild-type p53 consensus target sequences measured as increase in the activity of a reporter gene, as well as an increased protein level of p21 which is involved in regulation of cell cycle progression. Recently, it was shown that EGR-1 expression is elevated in cells expressing mutant p53 [17]. This protein was suggested to activate IGF-II, PDGF-A and PDGF-B involved in cell proliferation [27–29]. BCL-2, fibronectin and NF-xB associated with survival and cell differentiation [30–32] and PTEN as well as TNF-α involved in apoptosis [33,34]. The scFv ME1 was able to abrogate the mutant p53 “gain of function”, resulting in decreased levels of EGR-1 expression.

These results demonstrated that the scFv ME1 directed to the common epitope of mutant p53 is specific for the mutant form and restores wild-type properties to the mutant p53 protein. On that basis, we suggest that the use of site-directed antibodies may serve as a tool in p53-based cancer therapy.

References


