Targeting heterogeneous nuclear ribonucleoparticule A1 and A2 proteins by RNA interference promotes cell death in transformed but not in normal mouse cell lines

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Abstract
The heterogeneous nuclear ribonucleoparticule A1 and A2 proteins can bind to vertebrate single-stranded telomeric sequences. Moreover, changes in the levels of heterogeneous nuclear ribonucleoparticule A1 can influence telomere length in mouse and human cells. We have shown previously that the combined knockdown of A1 and A2 proteins in human transformed cells promotes apoptosis. In contrast, a similar reduction in A1 and A2 expression in normal mortal human cell lines does not induce cell death. Here, we show that a variety of mouse cell lines display a similar behavior on reduction of A1 and A2 protein levels using small interfering RNA. In addition, the expression of the mouse A1 cDNA protects human HeLa cells from apoptosis when human A1 and A2 proteins are targeted by RNA interference. Lastly, we show that knockdown of A1 and A2 expression also impairs the growth of a human transformed cell line that does not express telomerase. These results firmly establish A1 and A2 as proteins required for the viability of transformed murine and human cells, irrespective of the status of telomerase expression or the length of the double-stranded telomeric repeat. [Mol Cancer Ther 2004;3(10):1193–9]

Introduction
In cell lines established from mouse or humans, the heterogeneous nuclear ribonucleoparticule A1 and A2 proteins are among the most abundant nuclear proteins. A1 and A2 share 69% amino acid identity, and the related A1B and B2 proteins, respectively, represent their less abundant splice isoforms. Although the function of A1 and A2 in RNA processing events is well documented (reviewed in ref. 1), these proteins also bind with high affinity to telomeric ssDNA sequences (2–4). Importantly, mouse erythroblastic cells defective in A1 expression harbor short telomeres, which can be elongated by restoring expression of A1 (3). A similar result is obtained with UP1, a shortened derivative of A1 lacking the glycine-rich COOH-terminal domain that is important for the activity of A1 in alternative RNA splicing (3). Whereas the A1 and A2 proteins are expressed at high levels in transformed and proliferating human cancer cells, their expression is reduced and more restricted in normal human tissues (5–13). Remarkably, in a variety of human cancer cell lines, a concomitant RNA interference (RNAi)–mediated decrease of both A1 and A2 proteins elicits apoptosis even in p53-compromised cells (13). Moreover, knockdown of A1 and A2 expression in HeLa cells is accompanied by a reduction in the length of telomeric single-stranded G-tails, an event not observed when apoptosis is induced with staurosporine (13). In sharp contrast, normal human cell lines do not undergo programmed cell death when the levels of A1 and A2 proteins are reduced in an equivalent manner (13).

These observations suggest that, in human cancer cells but not in normal somatic cells, the A1 and A2 proteins play a role in the function of the telomeric cap that protect telomeres from degradation and prevents the end of chromosomes from being recognized as DNA breaks. All vertebrate species share identical telomeric repeat sequences (TAGGGT), but the actual length of the telomeric repeat tracts varies significantly. For example, mouse telomeric repeat tracts are generally much longer (20–100 kb) than their human counterparts (5–15 kb; ref. 14). Moreover, mouse somatic tissues express considerably higher levels of the enzyme telomerase than equivalent normal human tissues (15). If manipulating the levels and activity of A1 and A2 interfered directly with the distal telomeric cap structure, the eventual phenotypic outcomes should be independent of actual telomere length. The goal of the current study was therefore to examine whether mouse cell lines behave in a similar way when confronted to a reduction in A1 and A2 protein levels.

Using specific small interfering RNA (siRNA)–mediated RNAi to promote a reduction in A1 and A2 protein levels, we show here that several mouse cancer cell lines are highly sensitive to a concomitant reduction in A1 and A2 proteins. The specificity of cell death mediated by siRNAs targeting A1 and A2 was demonstrated by showing that the mouse A1 protein protects human cancer cells from apoptosis when these cells sustain a reduction in human A1 and A2 proteins. Notably, and in accordance with the results observed in human cells, normal mouse cell lines
in which levels of A1 and A2 have been reduced by identical RNAi procedures do not experience cell death. Therefore, the cellular consequences of reducing A1 and A2 protein levels are independent of the actual telomeric repeat tract length. These results corroborate the idea that the telomere capping function of A1 and A2 is conserved in transformed mouse and human cancer cells. The ability of normal somatic mouse cells to tolerate a reduction in A1 and A2 levels, as was also observed for normal human cells (13), suggests that telomere capping in normal cells involves different proteins than in transformed cells. These results are consistent with the limited current knowledge of vertebrate capping proteins. The results also strengthen the position of A1 and A2 as validated cancer targets.

Materials and Methods

Cell Cultures

4T1, F9, P19, J774A.1, NIH/3T3, and WI38VA13 cells were obtained from the American Type Culture Collection (Manassas, VA). Mouse embryonic fibroblasts (MEF) were prepared from 13- to 15-day-old embryos of BALB/c mouse obtained from Charles River Laboratories (Wilmington, MA). F9 cells were grown on gelatin-coated dishes in DMEM supplemented with 10% fetal bovine serum. 4T1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. 4T1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. P19 cells were grown in α-MEM supplemented with 10% fetal bovine serum. J774A.1, NIH/3T3, MEF, and HeLa S3 cells were grown in DMEM supplemented with 10% fetal bovine serum. WI38VA13 cells were grown in α-MEM supplemented with 10% fetal bovine serum, Earle’s salt, 1 mmol/L sodium pyruvate, and 0.1 mmol/L nonessential amino acids.

Selection of a Human Cell Line That Expresses the Mouse A1 Protein

HeLa S3 cells were transfected with a plasmid encoding a myc-tagged mouse A1 cDNA using DOSPER Liposomal Transfection Reagent (Roche Diagnostics, Laval, Quebec, Canada). Two days post-transfection, cells were reseeded at a lower density and 400 g/mL of Geneticin (Invitrogen Canada Inc., Burlington, Ontario, Canada) was added for positive selection of transfected cells. Clonal zones were individually reseeded in 24-well plate and screened for myc-A1 protein expression using an anti-myc antibody (see Fig. 5). In addition to the human A2 protein, the resultant positive clones therefore expressed the human and mouse A1 proteins.

siRNAs

Oligos used in this study were purchased from Dharmacon Research, Inc., (Lafayette, CO) as described previously (see ref. 13; Table 1).

siRNA Transfection

One day before transfection, exponentially growing cells were trypsinized and seeded into six-well plates. Transfections were done on cells using Oligofectamine (Invitrogen) or LipofectAMINE 2000 (Invitrogen) according to manufacturer’s instructions. Concentrations of 80, 40, and 20 nmol/L of total siRNAs were used for mouse, HeLa S3, and WI38VA13 cells, respectively.

Cell Growth and Viability Measurements

At the indicated times after the first transfection, both adherent and floating cells were harvested and counted. Cell viability was evaluated by trypan blue exclusion (13). Cell growth values for siRNA-treated cells were normalized relative to control treatment and were a mean of at least three independent experiments.

For DNA content analysis, both floating and adherent cells were recovered, fixed in 80% cold ethanol, incubated at room temperature for 5 minutes, and stored at −20°C. The cells were washed with PBS-A and treated with RNase A for 30 minutes at 37°C (20 μg RNase A, 5 mmol/L EDTA, and 0.5% bovine serum albumin in 1 mL PBS-A). The cells were stained with propidium iodide (50 μg/mL) for 5 minutes at room temperature and read on a FACScan using the CellQuest software (BD Biosciences, Mississauga, Ontario, Canada). For each sample, at least 10,000 events were analyzed for DNA content.

Table 1. Activity of siRNA

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence (AA-N19) mRNA Target</th>
<th>A1 and A2 mRNA Target</th>
<th>siRNA Activity</th>
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<tr>
<td></td>
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<td>Human</td>
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<tr>
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<tr>
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<tr>
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<td>Yes</td>
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<tr>
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<tr>
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</table>

*HeLa S3 cells.
†F9 cells.
*Occasional activity.
Whole cell extracts were prepared by lysing cells in 1× Laemmli sample buffer [10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 62.5 mmol/L Tris-HCl (pH 6.8), and 0.1% bromophenol blue]. Equal amounts of each sample (15–25 μg of total protein) were loaded onto a polyacrylamide gel. Western blotting was done according to standard protocols using the following dilution for primary antibodies: 1:6,000 for the anti-A1 and A2 antibodies (Telogene, Inc., Sherbrooke, Quebec, Canada), 1:100 for the anti-caspase-3 antibodies (AB3623, Chemicon, Temecula, CA), and 1:1,000 for anti-myc antibody (OP10, EMD Biosciences Inc., San Diego, CA).

Results

RNAi on Heterogeneous Nuclear Ribonucleoparticles A1 and A2 in Mouse Cells

To determine if heterogeneous nuclear ribonucleoparticle A1 and/or A2 expression in mouse cells is essential for cell growth, we used siRNAs to reduce the levels of A1 and/or A2 proteins in mouse cancer cell lines. Double-stranded siRNAs against A1 or A2 were introduced into the testicular embryonic carcinoma F9 cell line by performing two successive applications of siRNAs at a 24-hour interval. siRNAs hmA1-6 and hmA2-1, which have been shown previously to be active against human A1 and A2, respectively, match perfectly the sequence of the mouse A1 and A2 cDNAs (see Table 1). The combined application of siRNAs hmA1-6 and hmA2-1 promoted a marked reduction in the expression levels of both A1 and A2 proteins in the mouse cells (Fig. 1A). In contrast, a set of human-specific siRNAs, which contain mismatches when compared with mouse transcripts (hA1-1, hA1-5, and hA2-5), did not elicit a change in the abundance of the mouse A1 or A2 proteins (Table 1; Fig. 1A). As expected, the mutated siRNA hmA1-6M also did not elicit a reduction in the level of mouse A1 (Fig. 1A). Although the human-specific siRNA hA2-3 harbors one mismatch with the mouse A2 transcript (see underlined nucleotide in Table 1), it displayed activity in F9 cells on some occasions (data not shown). This positive result is not unexpected because the G-U bp that occurs at the mismatched position may still permit RNAi activity.

To assess whether the siRNA treatments affected the growth of F9 cells, we counted the total number of cells 72 hours following the first treatment with various siRNA mixtures (Fig. 1B). Cellular morphology was also assessed by microscopic examination (Fig. 1C). Individual or combined siRNA treatments with human-specific siRNAs did not affect cell growth (Fig. 1B). Likewise, mixtures that contained siRNAs targeting the mouse A1 or the A2 transcripts separately did not impact cell growth (Fig. 1B). In contrast, four different mixtures of siRNAs targeting both mouse A1 and A2 transcripts promoted a considerable drop in cell growth and F9 cells displayed an altered morphology (Fig. 1B and C). The ability of RNAi to reduce A1 and A2 levels and impede cell growth was also investigated in cell lines derived from other mouse cancers. We tested the mammary metastatic cancer cell line 4T1, the macrophage sarcoma cell line J774A.1, and the teratocarcinoma P19 cell line. Cell viability was measured 72 hours...
post-transfection as before. Similar to what was observed in F9 cells, treatment with individual siRNAs promoted a reduction in the targeted protein(s) (Fig. 2A), but only the combination of siRNAs targeting both mouse A1 and A2 affected the growth of 4T1, J774A.1, and P19 cells (hmA1-6/hmA2-1; Fig. 2B and C). The mutated hmA1-6 (hmA1-6M) alone or in combination with hmA2-1 did not elicit a reduction in A1, and the cells displayed normal growth and morphology (Fig. 2).

A trypan blue exclusion staining of treated cells indicated that the siRNA mixture that affected cell growth and morphology promoted cell death (data not shown). To assess whether cell death was occurring by apoptosis, we carried out a pro-caspase-3 cleavage assay as well as DNA content analysis (Fig. 3). Only the treatment with the pair of active siRNA (hmA1-6/hmA2-1) promoted the detection of the pro-caspase-3 cleavage product in the F9 cell line (Fig. 3A). The DNA content analysis done on F9 and 4T1 cells showed the characteristic sub-G1 increase expected for DNA fragmentation (Fig. 3B). We conclude that the rapid cell death of mouse cells induced by siRNAs against A1 and A2 occurs by apoptosis.

Nontransformed Mouse Cell Lines Are Resistant to the RNAi-Mediated Decrease in A1 and A2

To monitor the impact of a reduction in A1 and A2 levels on normal mouse cells, we used two cell lines: NIH/3T3 fibroblasts and mortal MEF cells. MEF cells were obtained from 13- to 15-day-old embryos of BALB/c mice. NIH/3T3 and MEF cells express A1 and A2 proteins at levels that are comparable with the levels detected in the mouse transformed cell lines used above. RNAi assays with siRNAs against A1, A2, or both promoted a reduction in the corresponding proteins that was equivalent to the reduction observed in similarly treated transformed cells (Fig. 4A). Although abrogating A1 and A2 expression had a small impact on cell growth, particularly for NIH/3T3 cells (Fig. 4B), the treatment did not affect cell morphology (Fig. 4C) or the cell fractionation profile (Fig. 4D). Thus, in contrast to transformed cells, the siRNA treatment was well tolerated by normal mouse somatic cells with no indication of cell death. The reason for the more noticeable reduction in the growth of NIH/3T3 cells is unknown, but it may be linked to the immortalization status of these cells. A similar growth reduction but no cell death was also observed with a human immortalized cell line (BJ-TIELF; ref. 13).

Protective Effect of Mouse A1 Expression on RNAi-Treated Human Cells

The above results paralleled and corroborated results previously obtained with human cells (13). Taken together, the data suggest a homologous essential function of mouse and human A1 and A2 in transformed cells. Although highly homologous in sequence, mouse and human A1 mRNAs do differ at various positions and siRNAs completely complementary only to human, mouse, or both can be derived (Table 1). Such common or species-specific siRNAs therefore provided the necessary tools to examine the specificity of the siRNA treatments. To assess whether in human cells the cell death phenotypes induced by siRNAs against hA1/hA2 can be rescued by expressing the mouse A1 protein, we carried out siRNA treatments of HeLa S3 cells engineered to express the mouse A1 cDNA. HeLa S3 cells were transfected with a plasmid expressing a myc-tagged mouse A1 cDNA and a clonal derivative was isolated. Expression of the myc-tagged mouse A1 was confirmed by performing a Western blot with anti-myc antibody (Fig. 5B). When this cell line is treated with a mixture of siRNAs that target the mouse and human A1 (siRNA hmA1-6; Table 1), as well as the human A2 (siRNA hmA2-1; Table 1), expression of all three proteins (human A1 and A2, mouse myc-tagged A1) was markedly decreased (Fig. 5B), cell growth and cell morphology were severely impaired (Fig. 5A and D), and cell death occurred as indicated by the detection of the apoptotic marker caspase-3 (Fig. 5C). In contrast, HeLa cells treated with siRNAs that only abrogated human A1 and A2 (hA1-1/hmA2-1) grew almost normally and did not display the phenotypes associated with cell death (Fig. 5A, C, and D). These data show that expression of the mouse A1 cDNA protects HeLa S3 cells from the deleterious effect of reducing the levels of human A1 and A2 proteins. Therefore, the cell death

Figure 2. siRNA treatment of 4T1, J774A.1, and P19 mouse cancer cell lines. A, 72 hours post-transfection, cells were harvested and A1 and A2 proteins were analyzed by Western blot using the anti-A1 and A2 antibody. B, effect of various siRNA treatments on cell growth (relative to control). White histograms, cells with an altered morphology. C, phase-contrast microscopy (×200 magnification) of 4T1, P19, and J774A.1 cells treated with siRNAs.

Mol Cancer Ther 2004;3(10). October 2004

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observed in previous experiments (13) is caused by the simultaneous reduction in A1 and A2 proteins and not through some nonspecific effects of the active pair of siRNAs or via targeting an unrelated RNA, which would carry a stretch of sequence complementary to the siRNAs.

Telomerase Activity Is Not Required for the siRNA-Induced Effect on Cell Growth

Whereas cell cycle–dependent activation of telomerase is a property of normal human somatic cells (16), uncontrolled activation of telomerase is a hallmark of the majority of human cancers. To investigate the contribution of telomerase expression in the siRNA-induced growth effect on cancer cells, we tested the impact of knocking down A1 and A2 expression in transformed cells that lack telomerase and maintain their telomeres through the Alternative Lengthening of Telomeres (ALT) pathway (17). The treatment of human WI38VA13 cells with the siRNA combination hmA1-6 and hmA2-1 promoted a reduction in the expression of A1 and A2 in WI38VA13 cells (Fig. 6) and strongly affected both cell growth and cell morphology (Fig. 6; data not shown). These results indicate that a marked impairment of cell growth elicited by a drop in A1 and A2 expression can occur in transformed cells that do not express telomerase.

Discussion

Previously, we have shown that a reduction in human A1 and A2 proteins elicited by the application of specific siRNAs promotes cell death by apoptosis in a variety of human cancer cell lines (13). In contrast, normal epithelial and fibroblastic human cell lines are resistant to a similar reduction in A1 and A2 proteins. Here, we show that, on a
similar treatment, four different cancer mouse cell lines and two normal mouse cell lines display phenotypes that parallel those of human cancer and normal cell lines, respectively, in an almost indistinguishable fashion. Furthermore, the effects mediated by the siRNAs are specific, because mutated or mismatched siRNAs against A1 or A2 did not promote the death of mouse cancer cells. Moreover, expressing the mouse A1 cDNA in HeLa cells protects them from the deleterious effect of a human-specific siRNA-mediated reduction in the levels of A1 and A2 proteins. This result confirms that the drop in A1 and A2 is intimately associated with the induction of apoptosis and is particularly important now that siRNA treatments have been documented to promote nonspecific effects on gene expression (18–20).

Our study suggests that the reduction in A1 and A2 proteins affects a common mechanism in a large variety of different mouse and human cancer cells. We believe that this common process is telomeric capping for the following reasons. First, the A1 and A2 proteins bind with high affinity to single-stranded telomeric sequences in vitro (2–4). Second, both the telomeric repeat sequence TAGGGT and the amino acid sequence of the A1 and A2 proteins are perfectly conserved between mouse and human. Third, a reduction in A1 protein alone in mouse cells is associated with telomere shortening, and restoring or overexpressing A1 in mouse and human cancer cells, respectively, promotes telomere elongation (3). Furthermore, expression of UP1, a shortened version of A1 that behaves in a dominant-negative manner in alternative splicing assays (21), also promoted telomere elongation in mouse and human cells (3). Fourth, the effects observed are independent of overall double-stranded telomeric repeat length. Fifth, we have shown that the siRNA-mediated knockdown of A1 and A2 in HeLa cells promotes a reproducible reduction, but not a complete loss, in the length of the single-stranded G-rich extensions, an event that is not observed when apoptosis is induced by staurosporine (13). We have been unable to detect similar changes in G-tails on telomeres constitutes but one of many possible consequences of telomere uncapping. For example, altering the capacity of cells to form T-loops, a particular displacement loop conformation at telomeres (22), may disrupt the...

Figure 5. Protective effect of mouse heterogeneous nuclear ribonucleoparticle A1 expression in HeLa S3 cells challenged with human-specific siRNAs against A1 and A2. A, 96 hours after the first transfection, cells were harvested and counted. Columns, relative to the counts obtained with the control sample (RU). White histogram, cells with an altered morphology. B, Western analysis of A1 and A2 expression. 96 hours after siRNA treatment, the A1 and A2 proteins were revealed with the anti-A1 and A2 antibody. The myc-A1 protein was revealed with an anti-myc antibody. Further analyses indicated that myc-A1 comigrates with the low abundance human B1 protein (data not shown). *, position of the myc-A1 and B1 proteins. Based on the intensity of this band when all human A and B proteins are targeted (hmA1-1 + hmA2-1), we estimate that myc-A1 may represent as much as 50% of the total amount of A1 proteins in the untreated HeLa S3 cell clone. C, a Western blot was done using the antibody against activated caspase-3. Ponceau S staining of the nitrocellulose membrane confirmed equal protein loading (data not shown). D, phase-contrast microscopy (×200 magnification) of cells treated with siRNAs.

Figure 6. Abrogating A1 and A2 expression in a telomerase-minus human transformed cell line. 96 hours post-transfection with various siRNA mixtures, WI38VA13 cells were harvested and counted (top). Western analysis (bottom) was carried out with the anti-A1 and A2 antibody.
Telomeric cap and induce apoptosis even in the absence of actual losses of the G-tails. The fact that mouse and human normal somatic cells are resistant to similar reductions in A1 and A2 levels suggests that either the structure of the telomeric cap in such cells is different from that of cancer cells or their capacity to form a protective cap is less dependent on A1 and A2 levels. Nevertheless, formally we cannot exclude the possibility that the phenotype observed is mediated through a telomere-independent mechanism.

Because the majority of cancer cell lines express telomerase, our results with the mouse and human cell lines raised the issue of whether telomerase expression is important or even required for cell death when mediated by the A1 and A2-specific siRNAs. Such a requirement could exist because heterogeneous nuclear ribonucleoparticule A1 can interact simultaneously with telomeric ssDNA and telomerase RNA in vitro (23). However, several observations suggest that telomerase activity makes no contribution to the phenotypic effect associated with reduced A1 and A2 protein levels. First, we have determined that the transformed human cell line WI38VA13, which uses a telomerase-independent mechanism to maintain stable telomere length (ALT pathway: refs. 24, 25), is equally sensitive to abrogation of A1 and A2 expression and undergoes rapid cell death on the application of A1 and A2-specific siRNAs. Second, A1 and A2 abrogation in a normal human cell line restored for telomerase expression (BJ-TIELF) does not promote cell death (13). Third, mouse NIH/3T3 and MEF cells are resistant to a knockdown of A1 and A2 mediated by siRNAs. These results indicate that telomerase expression is not required to elicit the phenotypes observed. Rather, they are consistent with the idea that the drastic induction of cell death on A1 and A2 targeting by RNAi is independent of the status of telomerase expression.

The differential impact of a knockdown of A1 and A2 in transformed and normal mammalian cell lines validates A1 and A2 as targets in cancer treatment. The expression profiles of A1 in mouse and A2 in rat are very similar to the expression profile of A1 and A2 in human tissues. Except for neurons, skin basal layer cells, and germ cells, which express high levels, most mouse and rat tissues express low or undetectable levels of A1 and A2 proteins (13, 26, 27). The identical response to RNAi-mediated reduction of A1 and A2 protein levels displayed by human and mouse cancer cell lines therefore suggests that the mouse should be an appropriate model organism for testing potential anticancer compounds targeting A1 and A2.

References
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