Small Interfering RNA-Mediated Reduction in Heterogeneous Nuclear Ribonucleoparticule A1/A2 Proteins Induces Apoptosis in Human Cancer Cells but not in Normal Mortal Cell Lines

Caroline Patry, Louise Bouchard, Pascale Labrecque, Daniel Gendron, Bruno Lemieux, Johanne Toutant, Ely Lapointe, Raymund Wellinger, and Benoit Chabot

Département de Microbiologie et d’Infectiologie. Faculté de Médecine. Université de Sherbrooke, Sherbrooke, Québec, Canada

ABSTRACT

To prevent their recognition as DNA breaks, the ends of linear chromosomes are organized into telomeres, which are made of proteins bound to telomere-specific, double-stranded repeats and to single-stranded DNA extensions, the G-tails. The mammalian heterogeneous nuclear ribonucleoparticule A1 and A2 proteins can bind with high affinity to such G-tails. Moreover, previous work established that in certain mouse cells a severe reduction in the level of A1 is associated with shortened telomeric repeat tracts, and restoring A1 expression increases telomere length. Here, we show that the expression of A1/A2 proteins is elevated in a variety of human cancers, whereas A1/A2 expression is lower or absent in normal tissues. To determine whether the status of A1/A2 proteins could be improved from cancer markers to cancer targets, we used small interfering RNA-mediated RNA interference to elicit a reduction in A1/A2 proteins in a variety of human cell lines. We show that this treatment provoked specific and rapid cell death by apoptosis in cell lines derived from cervical, colon, breast, ovarian, and brain cancers. Cancer cell lines that lack p53 or express a defective p53 protein were equally sensitive to a small interfering RNA-mediated decrease in A1/A2 expression. The reduction in A1/A2 levels in HeLa cells was associated with a change in the distribution of the lengths of G-tails, an event not observed when apoptosis was induced with staurosporine. Remarkably, comparable decreases in the expression of A1/A2 in several mortal human fibroblastic and epithelial cell lines did not promote cell death. Thus, manipulating the level and activity of A1/A2 proteins may constitute a potent and specific approach in the treatment of human cancers of various origins.

INTRODUCTION

The telomeres are essential for protecting chromosome ends from degradation, recombination, and fusion events. Telomeric DNA consists of a variable number of TAGGGT repeats in double-stranded form, followed by a single-stranded overhang of the guanine-rich strand, making up the 3’-end of the DNA (1). In human cells, the size of this overhang is estimated at 150–300 nucleotides, and at least a portion of this extension is thought to invade the proximal double-stranded telomeric DNA to form a T-loop (2, 3). Telomeric repeat DNA is bound by distinct protein complexes. For example, TRF1 and TRF2 bind directly to double-stranded telomeric DNA, interact with other proteins, and are important for telomere homeostasis (4, 5). Proteins that interact specifically with the terminal single-stranded extensions include the hnRNP A1 and A2 proteins (6–8), as well as the hPot1 protein discovered recently (9, 10). Finally, the ribonucleoprotein–enzyme telomerase interacts with the very terminus of the single-stranded telomeric repeats and extends the G-rich strand, a process that helps to solve the end-replication problem. However, although an absence of telomerase can lead to a gradual loss of telomeric sequences and eventual cell division arrest, replicative senescence is associated more with changes in the terminal capping structure itself than with the overall length of telomere repeat DNA (11). Consistent with this view, a loss of the single-stranded G-rich extension has been shown recently to correlate with senescence in human fibroblasts (12, 13).

Current cancer models for human cells postulate that telomeric decay imposed by active cell growth in the absence or low levels of telomerase successively triggers p53-dependent cell-cycle arrest, escape from the arrest, and continued divisions that increase the frequency of dicentric chromosome formation (14, 15). The resulting genetic instability may then promote mutagenic events until mutant cells that are able to maintain stable telomeres emerge and develop into a tumor. In ~85% of all of the tumors, stabilized telomeres are thought to be a direct consequence of the reactivation of the telomerase enzyme (16). However, distinct mechanisms involving other pathways (e.g., Alternative Lengthening of Telomeres or ALT) have also been uncovered (17, 18). Therefore, the presence of telomeric repeats for the capping function is absolutely essential for the growth of cancer cells, irrespective of their origin.

Many studies aimed at reversing the neoplastic phenotype of cells have targeted the activity of proteins involved in telomere biogenesis. For example, the expression of a catalytically inactive form of telomerase in human cancer cell lines promotes telomere shortening, ultimately leading to growth arrest and cell death (19–21). The use of telomerase inhibitors to promote telomere shortening in cancer cells is also being pursued (22–26). Proteins involved in the capping function of telomeres represent interesting targets as well (27, 28), and would also include proteins that recognize the single-stranded G-rich telomeric extensions. It is conceivable that approaches that will interfere with the capping function of telomeres in cancer cells may lead to rapid cell growth arrest and cell death. For example, the double-stranded DNA binding telomeric protein TRF2 may play a role in this capping function based on its role in T-loop formation and in the ability of a dominant-negative version of TRF2 to promote chromosome fusions and rapid p53-dependent apoptosis (5).

hnRNP proteins are among the most abundant nuclear proteins in mammalian cells. There are >20 hnRNP proteins, all of which can associate with precursor mRNAs, and many influence pre-mRNA processing and other aspects of mRNA metabolism and transport (29). The best-characterized protein of the group is hnRNP A1 (hereafter referred to as A1). A1 also binds with high affinity to telomeric single-stranded DNA sequences (7, 30) and can interact simultaneously with telomerase RNA in vitro (31). Importantly, defective A1 expression in mouse erythroleukemic cells results in short telomeres, which can be lengthened by restoring normal levels of A1 or by expressing UP1, a smaller version of A1, which lacks the A1 splicing function (7). Overexpressing A1 also elicits telomere elongation in...
human HeLa cells (7). A close homologue of A1, the hnRNP A2 protein (referred to as A2), shares 69% amino acid identity with A1. Although A2 as well can bind specifically to single-stranded telomeric sequence in vitro (8), its role in telomere biogenesis in vivo has not yet been confirmed. For both A1 and A2, less abundant splice variants have been described (A1B and B1, respectively). Interestingly, A1 expression is high in proliferating and transformed cells (32). A1 is overexpressed in colon cancers (33) and in mononuclear cells from leukemia patients (34), whereas the A2/B1 proteins have been used as early markers for the detection of lung and pancreatic cancers (35–40).

The purpose of this study was to further examine the relationship between A1/A2 expression and different types of human cancers. Immunohistochemistry with A1/A2 antibodies revealed a strong correlation between A1/A2 expression and cancer cells, with little expression in normal cell types. In addition, we used RNAi (41) to elicit a reduction in the level of A1/A2 proteins in human cell lines. The results show that the combined reduction in A1 and A2 expression promotes apoptosis in all of the cancer cell lines tested. In sharp contrast, a similar decrease in A1/A2 protein levels in normal mortal cell lines has no significant effect on cell growth. These results are consistent with the view that the A1/A2 proteins are functional homologues playing a role as mammalian telomeric capping factors. Therefore, abrogating A1/A2 expression would represent a powerful and specific approach to prevent the growth of cancer cells.

MATERIALS AND METHODS

Anti-hnRNP Antibodies. Rabbit polyclonal sera raised against a peptide unique to the hnRNP A1 protein (ASASSSQGR, anti-A1) or against a peptide common to both hnRNP A1 and A2 proteins (KEDTVEHHLRDYFYE, anti-A1/A2) were used for the immunohistochemical studies. Peptide synthesis and antibody production was carried out initially by the Service de Séquence de Peptide de l’Est du Quebec, (CHUL, Ste-Foy, Quebec, Canada). The specificity of each serum was confirmed by ELISA and Western analyses (Fig. 1).

Immunohistochemistry. The normal tissue screen was performed on 10 different normal human tissues using both sera. Two different sections of the same tissue sample were treated independently with each serum. Three different samples per cancer type were screened using the anti-A1 and the anti-A1/A2 sera. Immunohistochemistry was conducted by LifeSpan BioSciences Inc. (Seattle, WA). Briefly, serial dilution studies demonstrated that on paraffin-embedded, formalin-fixed tissues, the highest signal-to-noise ratios for the anti-A1 antibody was obtained at dilutions of 1:100 and 1:250, and for the anti-A1/A2 antibody at dilutions of 1:1000 and 1:2000. These antibodies were used as primary antibodies, and Vector antirabbit secondary antibody (BA-1000), Vector ABC-AP kit (AK-5000) with a Vector Red substrate kit (SK-5100) were used to produce a fuchsia-colored deposit. Tissues were also stained with a positive control antibody (CD31) to ensure that the tissue antigens were preserved and accessible for immunohistochemical analysis. The negative control consisted of performing the entire immunohistochemistry procedure on adjacent sections in the absence of primary antibody. Slides were imaged using a DVC 1310C digital camera coupled to a Nikon microscope.

Cell Cultures. HeLa S3, HCT 116, HT-1080, MCF-7, and CCD-18Co cells were obtained from the American Type Culture Collection. BJ foreskin normal fibroblasts were kindly provided by James Smith (Baylor College of Medicine, Houston, TX). HIEC cells were provided by Jean-François Beaulieu (Université de Sherbrooke, Quebec, Canada). PA-1 and SK-OV-3 cells were obtained from Claudine Rancourt (Université de Sherbrooke). U-373 MG and BJ-TIELF cells were a gift from David Fortin (Université de Sherbrooke) and Sam Benchimol (Ontario Cancer Institute, Toronto, Ontario, Canada), respectively. HCT 116 p53−/− were a gift from Bert Vogelstein and Kenneth W. Kinzler (Johns Hopkins Medical Institute, Baltimore, MD). HeLa S3 and U-373 MG cells were grown in DMEM supplemented with 10% FBS. HCT 116 and HCT 116 p53−/− cells were grown in McCoy’s 5A medium supplemented with 10% FBS. BJ and BJ-TIELF cells were grown in α-MEM supplemented with 10% FBS. HIEC cells were grown in Opti-MEM I supplemented with 5% FBS. PA-1 and SK-OV-3 cells were grown in DMEM-F12 supplemented with 10% FBS. MCF-7 cells were grown in αMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, and 10 μg/ml bovine insulin. HT-1080 and CCD-18Co cells were grown in α-MEM supplemented with 10% FBS, Earle’s salt, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids.

siRNAs. Oligos were purchased from Dharmacon Research, Inc. (Lafayette, CO). Sequences were selected from the targeted mRNA sequences and submitted to a BLAST search to ensure that only one human gene was targeted. Seven siRNAs targeting the human A1 mRNA (GenBank accession no. NM_002136) were tested. They covered nucleotides 107–127 from the start codon (A1−1), 135–155 (A1−2), 154–174 (A1−3), 217–237 (A1−4), 404–424 (A1−5), 601–621 (A1−6), and 757–777 (A1−7). Nucleotides 117 and 118 in
A1–1 were changed to CG to yield A1–1M. Five siRNAs were directed at the hnrnPA2 mRNA (GenBank accession no. NM_002137) and were from nucleotides 48–68 (A2–1), 57–77 (A2–2), 298–318 (A2–3), 615–635 (A2–4), and 922–942 (A2–5). Before transfection, siRNA duplexes were prepared by annealing the complementary oligos. The final concentration of the siRNA duplex was 50 μM in 20 mM KCl, 6 mM HEPES-KOH (pH 7.5), and 0.2 mM MgCl2.

siRNA Transfections. The transfection procedure described below indicated that typically at least 80% of the cells took up a fluorescent oligo. The day before transfection, exponentially growing cells were trypsinized and seeded into six-well plates. Transfection was performed on 30–50% confluent cells using Oligofectamine (Invitrogen) according to the manufacturer’s instructions and at the indicated total siRNA concentrations: HeLa S3 (80 nM), HCT 116 (60 nM), HCT 116 p53−/− (40 nM), HT-1080 (20 nM), PA-1 (10 nM), U-373 MG (10 nM), SK-OV-3 (40 nM), MCF-7 (80 nM), HIEC (80 nM), BJ (80 nM), BJ-TIELF (80 nM), and CCD-18Co (80 nM). Briefly, the siRNAs (10 μM) were mixed with 175 μL of OPTI-MEM-I (Invitrogen), whereas Oligofectamine was mixed with 1 μL of OPTI-MEM-I (Invitrogen), whereas Oligofectamine was mixed with 1 μL of OPTI-MEM-I (Invitrogen), whereas Oligofectamine was mixed with 1 μL of OPTI-MEM-I (Invitrogen), whereas Oligofectamine was mixed with 1 μL of OPTI-MEM-I (Invitrogen). The transfection reagent and the siRNAs were then mixed and incubated at room temperature for 20 min before being applied to cells. Fresh medium was added, and a second transfection at the same concentration of siRNAs was conducted 24 h later. At least three independent experiments were carried out for each cell line, and typical results are shown.

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 dye exclusion. The number of population doublings after transfection was calculated for each sample using the equation: PD = \log(N/N0)/\log 2, where N is the number of cells at the end of the experiment and N0 equals the number of cells at the beginning of the experiment. TUNEL labeling was performed using the ApopTag kit (Intergen; S7110), according to the manufacturer’s instructions. Propidium iodide (1 μg/mL) was used as a nuclear counterstain to visualize the whole cell population. Cells were visualized by fluorescence microscopy.

For DNA content analysis, both floating and adherent cells were recovered, fixed in 80% cold ethanol, incubated at room temperature for 5 min, and stored at −20°C. The cells were washed with PBS A and treated with RNase A for 30 min at 37°C (20 μg RNase A, 5 mM EDTA, and 0.5% BSA in 1 mL PBS A). The cells were stained with propidium iodide (50 μg/mL) for 5 min at room temperature and read on a Becton Dickinson FACSscan using the CellQuest software. For each sample, at least 10,000 events were analyzed for DNA content.

Western Blotting. Whole cell extracts were prepared by lysing cells in Laemmli sample buffer [1× = 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 62.5 mM Tris-HCl (pH 6.8), and 0.1% bromphenol blue]. Equal amounts of each sample (15 μl of each sample using the equation: PD = \log(N/N0)/\log 2, where N is the number of cells at the end of the experiment and N0 equals the number of cells at the beginning of the experiment. TUNEL labeling was performed using the ApopTag kit (Intergen; S7110), according to the manufacturer’s instructions. Propidium iodide (1 μg/mL) was used as a nuclear counterstain to visualize the whole cell population. Cells were visualized by fluorescence microscopy.

For DNA content analysis, both floating and adherent cells were recovered, fixed in 80% cold ethanol, incubated at room temperature for 5 min, and stored at −20°C. The cells were washed with PBS A and treated with RNase A for 30 min at 37°C (20 μg RNase A, 5 mM EDTA, and 0.5% BSA in 1 mL PBS A). The cells were stained with propidium iodide (50 μg/mL) for 5 min at room temperature and read on a Becton Dickinson FACSscan using the CellQuest software. For each sample, at least 10,000 events were analyzed for DNA content.

Western Blotting. Whole cell extracts were prepared by lysing cells in Laemmli sample buffer [1× = 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 62.5 mM Tris-HCl (pH 6.8), and 0.1% bromphenol blue]. Equal amounts of each sample (15–25 μg of total protein) were loaded onto a polyacrylamide gel. Western blotting was performed according to standard protocols using the following dilutions for primary antibodies: 1:5000 for the anti-A1/A2 antibodies, 1:500 for the anti-PARP antibodies (Biosource; AHF0262), 1:100 for the active caspase-3 antibodies (Chemicon; AB3623), and 1:500 for the anti-caspase-3 antibody (Biosource; AHZ0052).

Telomere G-Tail Extension Analysis (T-OLA). The T-OLA assay was carried out as described (42). For the staurosporine experiment, cells were treated for 28 h with staurosporine at a concentration of 1 μM at a final concentration of 1.0% DMSO. Control cells were treated with DMSO 1.0%. Briefly, genomic DNA was prepared by standard cell lysis protocols. Oligo (CCCTAA)n was end-labeled and phosphorylated by T4 polynucleotide kinase in the following reaction mixture: 0.16 μM of oligo, 1.6 μM of [γ-32P]ATP (3000 Ci/mmol, 10 μCi/mL), 70 mM Tris (pH 7.6), 10 mM MgCl2, 5 mM DTT, and 20 units T4 polynucleotide kinase in a final volume of 50 μL. The reaction was allowed to proceed for 40 min at 37°C. Then 1 μL of 0.1 M ATP and an additional 10 units of kinase were added before another 20-min incubation period. The enzyme was then heat-inactivated at 65°C for 20 min. The oligo was precipitated with ethanol and dissolved in water. Hybridization was conducted in a 20 μl volume containing 10 μg of denatured DNA, 0.5 pmol of oligo, 20 mM Tris (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD, and 0.1% Triton X-100 at 50°C for 12 h. Forty units of Taq ligase (New England Biolabs) and 2 μl of fresh 10 mM NAD stock were added, and the ligation reaction was allowed for 5 h at the same temperature. Reactions were ended by adding 30 μl of water and by phenol-chloroform extraction. Samples were ethanol-precipitated and dissolved in 6 μl of 10 mM Tris (pH 8.0)–1 mM EDTA buffer. Three μl of each reaction were run on a 6%–7% polyacrylamide gel, denatured by heating at 90°C, and quenched on ice before loading onto an 8% acrylamide-urea gel. Gels were exposed, and ligation products were quantified using Quantity One software (Bio-Rad). The value of the intensity of each band in the ladder of ligation products was normalized for the number of concatenated oligo in the product. The values obtained for ligation products containing 3 and 4 oligos were then grouped and compared with products containing 5 and more oligos by plotting their abundance relative to the total abundance of all of the selected products. This procedure allows for a clearer visualization of the loss of longer ligation products.

RESULTS

hnRNP A1/A2 Expression in Cancer and Normal Tissues. We used rabbit polyclonal antibodies to investigate the expression of hnRNP A1 and A2 in various human cancer biopsies and normal cell types. Immunohistochemistry was performed with the anti-A1 antibody, which recognizes the A1 and A1B proteins, and with the anti-A1/A2 antibody, which recognizes the A1/A1B/A2/B1 proteins (Fig. 1A). For each cancer, three samples from different patients were used. High levels of nuclear A1/A2 expression were observed in all three of the biopsies from breast, small cell lung, and ovarian carcinomas (Table 1; Fig. 1). For lung adenocarcinomas, pancreas carcinomas, and skin melanomas, two biopsies scored as high, and one sample scored as moderate. Two biopsies of prostate carcinomas were strongly positive for nuclear A1/A2 expression, but one biopsy remained negative. Thus, most cancer tissues examined displayed relatively strong levels of nuclear A1/A2.

The expression pattern of nuclear A1/A2 in normal human tissues was considerably different (Table 2; Fig. 1). Except for the basal layer of skin tissue, which expressed high-level of A1/A2, most other normal tissues examined expressed low to undetectable levels of A1/A2. Occasional A1/A2 expression was noted in some neurons, and significant expression was detected in kidney epithelia and endothelium, in bile duct, in intestinal macrophages, in lymphocytes, and in mesothelium of the spleen. The higher expression of the A1/A2 proteins in the nucleus of tumor cells as compared with normal cells confirms A1/A2 as potentially broad markers for cancer detection. The binding A1/A2 to telomeric sequences and the implication of A1

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Sample</th>
<th>A1/A2 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>Colonic carcinoma</td>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>3</td>
<td>+/+</td>
</tr>
<tr>
<td>Small cell lung carcinoma</td>
<td>3</td>
<td>++</td>
</tr>
<tr>
<td>Ovary carcinoma</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>Pancreas carcinoma</td>
<td>2</td>
<td>++</td>
</tr>
<tr>
<td>Prostate carcinoma</td>
<td>3</td>
<td>++</td>
</tr>
<tr>
<td>Skin melanoma</td>
<td>1</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 1. hnRNP A1/A2 expression in cancer tissues

on May 24, 2017. © 2003 American Association for Cancer Research.
in telomere biogenesis raises the possibility that A1/A2 proteins may not only constitute useful cancer markers but may also play a crucial role in the maintenance of the transformed state, possibly as telomeric capping factors.

RNAi on hnRNP A1 and A2 in HeLa S3 Cells. If the A1/A2 proteins are involved in the formation of a telomeric cap, abrogating their expression could induce telomere uncapping, which in turn may promote cell growth arrest and rapid cell death (27). To test this hypothesis, we used siRNAs to promote a specific reduction in the level of A1 and/or A2 proteins in human cancer cells. Double-stranded siRNAs against A1 or A2 were individually introduced into HeLa S3 cells by performing two successive applications of siRNA (80 nM) at a 24-h interval. Seven different siRNAs targeting A1 and 5 siRNAs targeting A2 were tested. siRNA A1–1M is a version of A1–1 that contains two changes at adjacent positions (GC to CG) in the A1–1 sequence yielding an siRNA with two mismatches with respect to the wild-type A1 mRNA. Ninety-six h after the first transfection, total protein was isolated and the abundance of A1 and A2 proteins was assessed by Western analysis using the anti-A1/A2 rabbit polyclonal antibody. When compared with control extracts, the extracts from cells transfected with siRNAs A1–1, A1–2, A1–5, and A1–6 showed a marked reduction in the expression level of A1. Similarly, except for A2–4, all of the siRNAs against A2 promoted a strong decrease in the A2 protein (Fig. 2A). As expected, the mismatched siRNA A1–1M did not affect the level of A1.

Next, we assessed whether the treatment with siRNAs affected cell growth. Adherent and nonadherent cells derived from transfected and control HeLa S3 cultures were counted 96 h after transfection (Fig. 2B). We also assessed cellular morphology by microscopic examination (Fig. 2C). Individual siRNAs that decreased either A1 or A2 did not affect cell growth nor did they change cell morphology, even when tested at a concentration of 300 nm (Fig. 2, B and C; data not shown). Likewise, pairs of siRNAs that affected A1 or A2 alone did not affect cell growth or cell morphology (e.g., A1–6/A2–4; data not shown), and the A1–1M/A2–1 pair, which promotes a reduction in A2 but not A1, had no effect on growth and cell morphology (Fig. 2B). However, combinations of siRNAs that promoted a reduction in the abundance of both A1 and A2 (siRNAs A1–1/A2–1 and A1–5/A2–5; Fig. 2B and data not shown, respectively) had a major impact on cell counts, and cells displayed an altered morphology reminiscent of apoptotic cells. In some experiments, the reduction in cell growth was less apparent, but the majority of the cells were round and lost adherence (data not shown). We attribute these variations between experiments to differences in the timing of cell death. Trypan blue exclusion staining indicated that the majority of the cells in the active combinations of siRNAs always yielded a higher number of dead cells (data not shown). For HeLa S3 cells, we typically used a concentration of 80 nm of siRNAs (40 nm of each siRNA when a mixture of two was used). Although mixtures of siRNAs were also active in HeLa S3 cells at 20 nm, lower concentrations did not reduce cell viability. Finally, we note that a 50% decrease in the intensity of both A1 and A2 bands was almost invariably associated with massive cell death.

To confirm that cell death was occurring by apoptosis, we carried out several indicative assays, including PARP and procaspase-3 cleavage assays, as well as DNA content analysis (Fig. 3). Only the treatment with the pair of active siRNAs against A1 and A2 promoted procaspase-3 and PARP cleavage (Fig. 3, A and B, respectively). We also carried out a TUNEL assay, which specifically stains apoptotic cells and which indicated that >70% of the HeLa cells were TUNEL-positive when treated with the A1–1/A2–1 siRNAs, as compared with <0.1% when the treatment was performed with the control A1–1/A2–1 combination (Fig. 3C). The DNA content analysis showed the characteristic sub-G1 increase because of DNA fragmentation with the pair of A1–1/A2–1 siRNAs but not with the control A1–1/A2–1 siRNAs (Fig. 3D). Thus, all of the tests support the conclusion that a reduction in A1/A2 proteins in HeLa S3 cells promotes apoptosis.

The rapid cell death elicited by siRNAs targeting A1/A2 is consistent with the view that these proteins may function as telomeric capping proteins. If this is the case, one of the first consequences associated with a reduction in A1/A2 levels may be a shortening or alteration of the single-stranded G-rich extension at telomeres. To determine whether the siRNA treatment affected the single-stranded extensions, we performed a T-OLA in HeLa S3 cells treated with active and control siRNAs. When cells were mock-treated or treated with a control pair of siRNAs (A1–1/A2–1), the combined relative percentage of ligation products corresponding to five or more oligos is about the same as the percentage of ligation-products corresponding to 3 and 4 oligos (Fig. 4, A and B). However, the distribution of ligation products derived from cells treated with the active pair of siRNAs (A1–1/A2–1) is quite distinct: the percentage of ligation products with 5 or more oligos is now lower than the percentage shorter products (see Fig. 4A for two independent experiments). A similar result was observed when the analysis was performed 48 h after transfection (data not shown). Importantly, the length distribution of the G-rich extensions did not change when HeLa S3 cells were treated with staurosporine (Fig. 4B), an inducer of apoptosis.
Impact of A1/A2-Targeted RNAi in Other Cancer Cell Lines.

The ability of RNAi to promote a reduction in A1 and A2 and to affect cell viability was also investigated in cell lines derived from different human cancers. We first tested the colorectal carcinoma cell line HCT 116 and a derivative that suffered the genetic ablation of the p53 alleles (HCT 116 p53<sup>-/-</sup>). siRNAs mixtures were applied to HCT 116 and HCT 116 p53<sup>-/-</sup> cells as above. Cell viability was measured 72-h after transfection. Similar to what was observed for HeLa S3 cells, treatment with individual siRNAs promoted a reduction in the targeted protein (Fig. 5A), but only the combination of siRNAs targeting both A1 and A2 affected the growth and morphology of HCT 116 cells (Fig. 5, B and C). The mismatched A1–1M/A2–1 siRNAs promoted a reduction in A2 only and did not affect cell counts or cell morphology. The apoptotic phenotype obtained with the pair of siRNAs that affected both A1 and A2 was confirmed by testing for PARP and procaspase-3 cleavage (data not shown). Notably, the p53<sup>-/-</sup> version of HCT 116 was equally sensitive to abrogation of A1/A2 expression (Fig. 5B).

The RNAi-Mediated Reduction in A1/A2 Does Not Affect the Growth of Nontransformed Human Cell Lines.

To evaluate the impact of a reduction in A1/A2 levels in normal mortal cells, we used three cell lines: foreskin fibroblasts (BJ cells), colonic CCD-18Co myofibroblasts, and the epithelial intestinal cell line HIEC. We also used the BJ-TIELF cell line, which is an immortalized derivative of the BJ-line that expresses the catalytic component (human telomerase reverse transcriptase) of the human telomerase (44, 45). All of these cells express hnRNP A1/A2 proteins (Fig. 7A). We noted that the immortal BJ-TIELF cell line expresses more A1/A2 than earlier passages of BJ cells (data not shown), consistent with the view that A1/A2 expression drops as mortal cells approach senescence (46). RNAi assays with siRNAs targeting A1, A2, or both promoted a reduction in A1/A2 expression correlated with a reduction in cell growth and a change in cell morphology characteristic of apoptosis (Fig. 5, B and C).

Additional cancer cell lines that were tested include an ovarian carcinoma cell line (PA-1), a metastatic ovarian carcinoma cell line (SK-OV-3), a glioblastoma cell line (U-373 MG), and a breast carcinoma cell line (MCF-7; Fig. 6). Notably, the SK-OV-3 cell line is p53 null, whereas U-373 MG cells express a mutated form of p53 (40, 43). In all of the cases, only the treatment with the pair of siRNAs A1–1/A2–1 elicited a marked reduction in the expression of A1 and A2, and this reduction was accompanied by a reduction in cell growth and a phenotypic change characteristic of apoptosis (Fig. 6; data not shown).

The RNAi-Mediated Reduction in A1/A2 Does Not Affect the Growth of Nontransformed Human Cell Lines. To evaluate the impact of a reduction in A1/A2 levels in normal mortal cells, we used three cell lines: foreskin fibroblasts (BJ cells), colonic CCD-18Co myofibroblasts, and the epithelial intestinal cell line HIEC. We also used the BJ-TIELF cell line, which is an immortalized derivative of the BJ-line that expresses the catalytic component (human telomerase reverse transcriptase) of the human telomerase (44, 45). All of these cells express hnRNP A1/A2 proteins (Fig. 7A). We noted that the immortal BJ-TIELF cell line expresses more A1/A2 than earlier passages of BJ cells (data not shown), consistent with the view that A1/A2 expression drops as mortal cells approach senescence (46). RNAi assays with siRNAs targeting A1, A2, or both promoted a reduction in the corresponding proteins that was comparable with the drop obtained with cancer cell lines (Fig. 7A). In contrast to cancer cells, however, all of the mortal cell lines tolerated well a reduction in A1/A2, and the
treatment had little effect on cell growth and morphology (Fig. 7, B and C). Even the growth of the immortal but nontransformed BJ-TIELF cells was not affected by a strong reduction in A1/A2 (Fig. 7A). In all cases, cell cycle analysis of the DNA content indicated no significant increases in sub-G1 DNA content (Fig. 7D; data not shown). However, in experiments using BJ-TIELF cells, we occasionally noted a slight increase in the G2-M population (Fig. 7D). We also performed the T-OLA assay on DNA derived from CCD-18Co cells that were mock treated, and treated with.

Fig. 3. Apoptosis in HeLa S3 cells. A, HeLa S3 cells were transfected as described earlier, and cells were harvested 96 h after the first transfection. The Western blot was probed with a mixture of monoclonal antibodies that recognize both the M<sub>r</sub> 33,000 inactive procaspase-3 and the activated M<sub>r</sub> 20,000 form found in apoptotic cells. B, the same Western analysis as shown in A but probed with an antibody that recognizes the PARP enzyme, which is a substrate for the activated caspase-3 (52, 53). C, HeLa S3 cells were transfected with the indicated siRNAs or treated with 1 μM staurosporine. Ninety-six h after the first transfection or 15 h after staurosporine treatment, TUNEL labeling was performed. Propidium iodide was used as a nuclear counterstain to visualize the whole cell population. Apoptotic nuclei were labeled with fluorescein and appeared green, whereas living cells were stained red. Note that when cells are treated with both siA1 and siA2, there is a major decrease in the cell number as observed previously and that most of the remaining cells are positive for TUNEL labeling. D, siRNA-treated cells were fixed and stained with propidium iodide before DNA content analysis by cytometry. n refers to the haploid DNA content. Note the appearance of sub-G1 DNA, associated with apoptosis, in HeLa S3 cells. siA1, A1–1; siA2, A1–2; siA1M, A1–1M.

Fig. 4. T-OLA assay to measure the distribution of lengths of telomeric G-tails. A, 72 h after the first transfection, HeLa S3 cells were harvested and cellular DNA was extracted, and a T-OLA assay was performed. The results of two independent experiments are shown, the gel on the left depicting the results obtained in experiment 1. B, HeLa S3 cells were treated with staurosporine/DMSO or DMSO alone for 28 h before performing the T-OLA assay. S, staurosporine-treated cells; D, DMSO-treated cells. All oligo ligation assays were performed using 10 μg of cellular DNA and ligation products were resolved on a sequencing gel, detected by autoradiography and quantitated as described in “Materials and Methods.” The numbers on the right of the gels indicate the number of oligos in some ligation products. Values for ligation products representing the ligation of 3 and 4 oligos or 5–13 oligos were pooled and plotted as frequency (in percentages). siA1, A1–1; siA2, A1–2; siA1M, A1–1M.
control and active pairs of siRNAs (A1–1M/A2–1 and A1–1/A2–1, respectively; Fig. 7E). Consistent with the results showing no cell mortality on treatment with the active pair of siRNAs, there was no discernible difference in the T-OLA products between the three treatments. We conclude that in contrast to cancer cell lines, mortal human cell lines tolerate well the reduction in A1/A2 proteins imposed by RNAi.

DISCUSSION

The successful treatment of cancer rests on the identification of targets that are specifically expressed in cancer cells and also play an important role in promoting or allowing unlimited cell division. Although interesting targets with such properties have been identified in cancer cells of different origins, there are few examples of factors that play important roles in many types of cancers. Here, we present evidence that the hnRNP A1/A2 proteins could represent potential new and broad targets against cancer.

First, we report that the expression of A1/A2 is elevated in the nuclei of cells derived from a variety of cancers (Fig. 1; Table 1). Moderate to high levels of hnRNP A1 proteins were detected in breast, prostate, ovary, pancreas, and skin cancer biopsies. These data confirm and extend previous studies that have documented the expression of A1 and A2/B1 proteins in colon and lung cancers, respectively (33, 35–38). Most significantly, however, we show that the level of A1 and A2 is comparatively lower, and in many instances virtually absent, in normal somatic tissues, although A1/A2 expression can be detected in some specific cell types (Fig. 1; Table 2). Only the basal layer of the normal skin displayed high level expression of hnRNP A1/A2. However, it is likely that A1/A2 will be similarly expressed in other highly proliferative cell types.

Second, the preferential expression of A1/A2 in cancer cells led us to investigate the impact of a reduction in A1/A2 in different human cancer cell lines. Using siRNAs to promote specific decreases in A1/A2, we observed that such treatments elicited programmed cell death of HeLa cells. Remarkably, cancer cell lines derived from colon, ovarian, breast, and brain cancers were all similarly sensitive to a reduction in A1/A2 protein levels. Notably, a reduction in A1 alone or in A2 alone did not promote cell death. The importance of a combined reduction in both A1 and A2 suggests that A1 and A2 are functional homologues. Therefore, hnRNP A2 compensation would explain the survival of the mouse erythroleukemic CB3 cell line, which is severely deficient in A1 (7). Thus, in a situation where A1 and A2 are expressed in similar amounts, reducing the global level of A1/A2 by targeting either A1 or A2 does not affect cell growth, and only the
dual targeting of A1 and A2 allows a substantial decrease in the overall levels of A1/A2.

The rapidity with which cancer cells die after the treatment with siRNAs against A1 and A2 is consistent with the view that the A1/A2 proteins play a direct role as telomeric capping factors. In support of this view, we have shown that abrogating A1/A2 expression is accompanied by a change in the length distribution of telomeric single-stranded G-rich extensions (G-tails). Because this change can be detected 48–72 h after the first application of siRNAs, we hypothesize that it is a crucial event to trigger apoptosis in cells that rely on A1/A2 as capping factors. Consistent with this view, normal cells that support a significant reduction in A1/A2 do not show such a change in the size distribution of T-OLA products, suggesting that the A1/A2 proteins may be less important for capping of the G-tails in such cells. Notably, such changes in the integrity of G-tails are not observed when cells are treated with the apoptotic inducer staurosporine, suggesting that the degradation of G-tails is not an obligatory feature or consequence associated with apoptosis, and reinforcing the conclusion that the drop in A1/A2 is directly responsible for the changes at the G-tails. Although we favor the proposition that shortening of G-tails is an important step to initiate the apoptotic response, it remains possible that alterations in other A1/A2-mediated processes contribute or even cause apoptosis.

The observed induction of apoptosis in cancer cells appears to be independent of the status of p53 expression, because p53 null and p53 mutant cell lines are also very sensitive to RNAi against A1/A2. Notably, apoptosis triggered by a dominant-negative version of the telomeric factor TRF2 required a wild-type p53 protein (47). Therefore, the outcome of a drop in A1/A2 is more similar to the impact of telomerase inhibition on the growth of human cancer cells, which also is independent of the status of p53 (19).

In contrast to the situation in cancer cells, the siRNA-mediated reduction in A1/A2 levels in normal and mortal cell lines did not affect cell division and did not provoke cell death. The fact that these "normal" cell lines are more resistant to a loss of A1/A2 function is intriguing, and may indicate that the capping complex at telomeres is different between cancer and normal cells. This would not necessarily be unexpected, because the enzyme telomerase is expressed at appreciable levels in cancer, but not in normal cells. It has been shown recently that this enzyme localizes to the telomeres where it may play...
a role in the capping function (48–50). Therefore, telomerase expression and other mechanisms may lead to differences in the size of the single-stranded extensions, which may affect the identity and function of capping factors. We speculate that such differences include the A1/A2 proteins, because their abrogation by siRNA affects the length distribution of the G-tails. A similar decrease in the size of G-tails has been associated recently with replicative senescence of normal human cells in culture (12), and providing oligos complementary to G-tails to cells in culture induces damage-response pathways (51). These data point to a critical importance of the integrity of the G-tails for the capping function of telomeres. Therefore, our results are consistent with the hypothesis that abrogating the expression or inhibiting the function of cancer cell-specific G-tail binding proteins, such as A1/A2, could entail a severe reduction in the capping function and explain the cellular phenotypes observed in the present study.

In summary, our results demonstrate that a combined reduction of hnRNP A1/A2 causes programmed cell death in a variety of cancer cell lines, including p53-compromised cells. Our findings establish A1/A2 as potential drug targets in cancer therapeutics and provide a strong rationale for the development of strategies aimed at abrogating the expression or activity of hnRNP A1/A2 proteins in cancer cells. Such approaches should be particularly attractive, given that A1/A2 are expressed at low levels in normal tissues and that normal mortal cells can tolerate a reduction in A1/A2 levels without significant effects on cell viability.

ACKNOWLEDGMENTS

We thank D. Fortin, C. Rancourt, B. Vogelstein, and K. W. Kinzler for cell lines. We thank Silvia Bacchetti and Sam Benchimol for comments on the manuscript.

REFERENCES

Small Interfering RNA-Mediated Reduction in Heterogeneous Nuclear Ribonucleoparticule A1/A2 Proteins Induces Apoptosis in Human Cancer Cells but not in Normal Mortal Cell Lines

Caroline Patry, Louise Bouchard, Pascale Labrecque, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/22/7679

Cited articles
This article cites 51 articles, 26 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/22/7679.full.html#ref-list-1

Citing articles
This article has been cited by 37 HighWire-hosted articles. Access the articles at:
/content/63/22/7679.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.