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Knockdown of α spectrin in normal human cells by siRNA leads to chromosomal instability and decreased DNA interstrand cross-link repair

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Abstract

Nonerythroid α -spectrin (α IISp) is a structural protein involved in repair of DNA interstrand cross-links and is deficient in cells from patients with Fanconi anemia (FA), which are defective in ability to repair cross-links. In order to further demonstrate the importance of the role that α IISp plays in normal human cells and in the repair defect in FA, α IISp was knocked down in normal cells using siRNA. Depletion of α IISp in normal cells by siRNA resulted in chromosomal instability and cellular hypersensitivity to DNA interstrand cross-linking agents. An increased number of chromosomal aberrations were observed and, following treatment with a DNA interstrand cross-linking agent, mitomycin C, cells showed decreased cell growth and survival and decreased formation of damage induced α IISp and XPF nuclear foci. Thus depletion of α IISp in normal cells leads to a number of defects observed in FA cells, such as chromosome instability and a deficiency in cross-link repair.

Keywords

α -spectrin; siRNA knockdown; Chromosomal aberrations; DNA interstrand crosslinks; Damage-induced nuclear foci; XPF; DNA repair; Fanconi anemia

Spectrin is a structural protein, which in the cytoplasm of nonerythroid cells plays an important role in providing mechanical support for the cell membrane and is also involved in protein sorting, organelle and vesicle trafficking, neurite outgrowth and neurotransmitter release, as well as cell growth and differentiation [1-4]. We have demonstrated that nonerythroid α -spectrin (α IISp) is present in mammalian cell nuclei and is involved in repair of DNA interstrand cross-links [5-7]. It preferentially binds to purified DNA containing an interstrand cross-link; it co-localizes with the cross-link repair protein, XPF, and the Fanconi anemia protein, FANCA, in cross-link induced nuclear foci, and it is needed for the production of incisions produced by XPF at the site of an interstrand cross-link [5-7].

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We have shown that there is a deficiency in α IISp in cells from patients with Fanconi anemia (FA), a genetic disorder characterized by bone marrow failure, diverse congenital abnormalities, genomic instability and a marked predisposition to develop cancer [5,7-10]. Cells from patients with FA have a defect in ability to repair DNA interstrand cross-links [10-16] and this defect correlates with reduced levels of α IISp in these cells [5,14,16]. Based on these results, we have proposed a model for the role of α IISp in DNA repair. In this model, α IISp binds to DNA at sites of damage and acts as a scaffold to aid in the recruitment of repair proteins to these sites, thus enhancing the efficiency of the repair process [5-7,17]. In FA cells, decreased levels of α IISp leads to reduced binding of α IISp to damaged DNA and decreased recruitment of repair proteins to sites of damage, thus leading to decreased levels of DNA repair.

In order to further demonstrate the importance of the role that α IISp plays in normal human cells and in the repair defect in FA cells, studies were carried out to determine the effects of knocking down α IISp in normal cells by siRNA. The results showed that depletion of α IISp in normal cells by siRNA led to increased chromosomal instability, as ascertained by an increase in chromosomal aberrations/breaks. Damaging these cells with two different DNA interstrand cross-linking agents, mitomycin C (MMC) or 8-methoxypsoralen (8-MOP) plus UVA light, resulted in decreased cell survival and decreased formation of damage induced nuclear foci of proteins involved in DNA interstrand cross-link repair. Thus knocking down of α IISp in normal human cells leads to a number of the defects observed in FA cells, including chromosome instability and a deficiency in cross-link repair.

Materials and methods

Cell culture

Normal human lymphoblastoid cells (GM3299) (Coriell Institute for Medical Research) and FA complementation group A (FA-A) lymphoblastoid cells (HSC 72) (a gift from Dr. Manuel Buchwald, Toronto, Canada) were grown in RPMI 1640 medium (Hyclone) as previously described [5,7]. HeLa S3 cells (ATCC) were grown in F12K medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Hyclone).

siRNA analysis

siRNA against the N-terminal of α IISp, nucleotides 224-252, (AAGATTCCTATCGATTCCAGTCCTGTCTC) was purchased from Dharmacon and a control non-silencing siRNA was from Qiagen. Normal human lymphoblastoid cells were transfected with α IISp siRNA or non-silencing siRNA using Lipofectamine 2000 Transfection Reagent (Invitrogen) following the manufacturer's instructions. The siRNAs and Lipofectamine were combined in serum-free RPMI 1640 media and the serum levels were adjusted 4 hours post transfection to normal culture levels. HeLa cell transfections were carried out as above using Oligofectamine Transfection Reagent (Invitrogen). Cell survival was assessed using trypan blue exclusion. Cells were harvested at 24 and 48 hours after transfection and whole cell protein lysates prepared by resuspending the cells in Laemmli Sample Buffer plus β -mercaptoethanol (BioRad). Cells were viewed using a Leitz DMRB microscope (Leica) equipped with a DEI-750 analog camera (Optronics). Images were obtained using Image Pro-Plus 6.0 software (Media Cybernetics).

α IISp mRNA levels

Total RNA was extracted, as previously described [18], from siRNA transfected normal lymphoblastoid cells using the RNeasy Mini Kit (Qiagen). RT-PCR was performed and the RT-PCR products were resolved by electrophoresis on 8% polyacrylamide gels, which were

stained with Syber Green (Sigma-Aldrich) and scanned using a FluorImager (Molecular Dynamics).

Immunoblotting

For analysis of levels of α IISp in the siRNA transfected cells, whole cell lysates were subjected to SDS-PAGE and western blot analysis carried out as previously described [5,6]. Immunoblots were probed with anti- α spectrin (mAb 1622, Chemicon) or anti-tubulin (Santa Cruz Biotechnology). Immunoblots were developed using Pierce Ultra chemiluminescent substrate and exposed to X-ray film. Images were scanned and quantitation of band intensity was done using ImageQuant software (Molecular Dynamics).

Chromosome analysis

Normal lymphoblastoid cells transfected with either α IISp or nontarget siRNA were incubated for 46 hours at 37°C, 5% CO₂. Colcemid (Sigma-Aldrich) (0.1 μ g per ml) was added and incubation continued for 2 hours. The cells were harvested, swollen in hypotonic solution (0.075 M KCl) and fixed in 3:1 methanol/acetic acid. Slides were prepared and stained with either Wright's or Giemsa stain. At least 100 metaphases from either α IISp or nontarget siRNA transfected cells were scored for chromosomal abnormalities. Metaphases were viewed and images obtained as above.

Treatment of cells with DNA interstrand cross-linking agents and cell growth analysis

Normal lymphoblastoid cells transfected with either α IISp or nontarget siRNA were treated with MMC (0-500 nM) 24 hours after transfection. Cell growth was assessed at 24 hours after MMC treatment using trypan blue exclusion by comparing the number of live cells for each siRNA treatment to the original number of undamaged nontarget siRNA treated cells. In other experiments, these cells were treated with 3.5 μ M 8-MOP (Sigma-Aldrich) plus two dosages of UVA light, as previously described [7], and cell survival assessed 16 hours post damage.

Indirect immunofluorescence

Normal lymphoblastoid cells transfected with either α IISp or nontarget siRNA were treated with 400 nM MMC or 3.5 μ M 8-MOP plus UVA light 24 hours after transfection. The cells were harvested 16 hours post damage and examined for nuclear localization of α IISp and XPF using indirect immunofluorescence as previously described [7]. Primary antibodies used were anti- α spectrin or anti-XPF (sc-10161, Santa Cruz Biotech). Secondary antibodies were Alexafluor 488 goat anti-mouse IgG conjugate or Alexafluor 594 rabbit anti-goat IgG conjugate (Molecular Probes). Stained cells were viewed with a Leitz DMRB microscope at 440 $\times$ and images were captured using a DEI-750 analog camera [6]. Images were imported into a computerized imaging system using Image Pro-Plus 6.0 software and Adobe Photoshop CS.

Results

Transfection of cells with α IISp siRNA leads to reduction in expression of α IISp and decreased cell survival

Transient transfection of normal lymphoblastoid cells with α IISp siRNA led to a reduction in levels of α IISp. Immunoblot analysis showed that levels of α IISp were slightly decreased 24 hours after transfection with 25 pmol of siRNA and by 48 hours levels of α IISp were 40% of those in cells transfected with a nontarget siRNA (Figs. 1A and B). A similar reduction in levels of α IISp (36% of controls) was observed in HeLa cells 48 hours after transfection with 30 pmol of α IISp siRNA (Fig. 1C); cell survival in these cells was approximately 85% (Fig. 1D). Increasing the levels of α IISp siRNA above 30 pmol led to a dramatic decrease in cell

survival 48 hours after transfection (Fig. 1D), which correlated with decreasing levels of α IISp in these cells (Fig. 1E). Reducing levels of α IISp in the cells had no effect on the levels of FANCA (Fig. 1E). At 90 pmol α IISp siRNA, levels of α IISp in HeLa cells were totally depleted (data not shown) and there was no cell survival (Fig. 1D). In contrast, when HeLa cells were transfected with 90 pmol of nontarget siRNA, 84% of the cells survived (Fig. 1D). A similar correlation between decreasing levels of α IISp and cell survival was seen in normal lymphoblastoid cells (data not shown). Thus α IISp is essential for cell survival. For this reason, additional studies on these transfected cells examined cells 48 hours post transfection with 25-30 pmol of α IISp siRNA.

Levels of α IISp mRNA in the α IISp siRNA transfected cells were also decreased. RT-PCR analysis of total RNA from normal lymphoblastoid cells 48 hours post transfection with 25 pmol of α IISp siRNA showed that levels of α IISp mRNA were 38% of those of the nontarget siRNA transfected cells (Fig. 1F).

Transfection of cells with α IISp siRNA leads to changes in cell morphology

Morphological changes were observed in the α IISp siRNA transfected normal lymphoblastoid cells. Normal lymphoblastoid cells in culture are very pleiomorphic, with numerous pseudopodia (Fig. 2A). However, 48 hours after α IISp siRNA transfection, these cells appeared rounder and many were smaller with few if any pseudopodia (Fig. 2A). This change correlated with decreased levels of α IISp in these cells (Fig. 2B). These α IISp siRNA transfected normal cells morphologically resembled FA-A lymphoblastoid cells, which are typically round and smaller than normal cells (Fig. 2A). The reduction of levels of α IISp in FA-A cells (35% of normal) (Fig. 2C) are similar to those observed in the α IISp siRNA transfected normal cells (Fig. 2B).

Chromosomal abnormalities occur in normal cells transfected with α IISp siRNA

Metaphase spreads from normal lymphoblastoid cells were examined for chromosomal aberrations 48 hours after transfection with α IISp siRNA. The number of metaphases in these cells showing intrachromatid exchanges, radials/fusions and breaks was significantly greater than in the nontarget siRNA transfected cells (Fig. 3A and B). When the chromatid interchange figures (exchanges and radials/fusions) were converted into breaks, where one interchange was counted as one break [19], the total number of chromatid breaks was approximately 5 fold greater than in the nontarget siRNA transfected cells (Fig. 3B), which was a significant difference ($P < 0.01$). After damage with MMC (120 nM), the α IISp siRNA transfected cells showed a small (10%) increase in chromosomal aberrations, compared to nontarget siRNA transfected cells (data not shown).

Knockdown of α IISp in normal cells leads to increased sensitivity to DNA interstrand cross-linking agents

Knockdown of α IISp in normal cells led to a distinct hypersensitivity of these cells to DNA interstrand cross-linking agents. A marked decrease in cell growth was observed 24 hours after addition of MMC which was dependent upon the concentration of MMC used (Fig. 4A). At 500 nM MMC, the growth of the α IISp siRNA transfected cells was only approximately 50% of that observed for the nontarget siRNA transfected cells.

Knockdown of α IISp in normal cells also influenced cross-link induced nuclear foci formation. In nondamaged, nontarget transfected cells, both α IISp and XPF were present in the nucleus in a diffuse pattern (Fig. 4B). 16 hours after treatment of the nontarget siRNA transfected cells with MMC (400 nM), damage-induced α IISp and XPF nuclear foci were observed (Fig. 4B). These results are similar to those we described in non siRNA transfected lymphoblastoid cells in which we showed that 16 hours after treatment with a DNA interstrand cross-linking agent,

there is a peak in formation of damage-induced α IISp and XPF nuclear foci [7]. In contrast, in undamaged α IISp siRNA transfected cells the levels of α IISp but not XPF were significantly reduced (Fig. 4B), indicating that knockdown of α IISp does not affect the levels of XPF in these cells. 16 hours after treatment of the α IISp siRNA transfected cells with MMC (400 nM) there was a marked reduction in damage-induced α IISp and XPF nuclear foci with only a few foci observed (Fig. 4B). However, levels of XPF were not reduced in these cells (Fig. 4B).

Similar results were obtained for normal lymphoblastoid cells transfected with α IISp siRNA and treated with 8-MOP plus UVA light. 16 hours after treatment of the α IISp siRNA transfected cells with 8-MOP plus UVA, levels of α IISp were markedly reduced (Supplementary Fig. 1A). Survival of the α IISp siRNA transfected cells after damage was approximately 45% of that observed for the nontarget transfected cells (Supplementary Fig. 1B). A marked reduction in α IISp nuclear foci was also observed in α IISp siRNA transfected cells 16 hours after treatment with 8-MOP plus UVA light (Supplementary Fig 1C).

Discussion

We have previously shown that α IISp is present in the nucleus of normal human cells where it plays an important role in repair of DNA interstrand cross-links and that there is a deficiency in α IISp in FA cells which correlates with their cross-link repair defect [5,7,14,16]. The present studies showed that complete knockdown of α IISp is lethal, in keeping with reports that α IISp is an essential protein in the cell [20,21]. Therefore, in the present study levels of siRNA knockdown of α IISp were adjusted so that they were approximately 35-40% of normal, similar to those found in FA cells [5,14,16].

The present results showed that decreased expression of α IISp in normal cells by α IISp siRNA led to a number of changes in cellular morphology, chromosomal stability and response to DNA damage. Morphologically the cells, normally very pleomorphic with numerous pseudopodia, became rounder with few pseudopodia and more closely resembled FA-A cells in culture. This could indicate that α IISp, which is also present in the cytoplasm, is important for structural support of the cell membrane and contributes to the normal morphology of the cell. Spectrin has been shown in nonerythroid mammalian cells to be important in membrane structure and integrity and cell shape [1,2,22].

The present results also show that α IISp is important for chromosomal stability. Reduction in levels of α IISp to 35-40% of normal by siRNA led to a marked (5 fold) increase in chromosomal aberrations, which included intrachromatid exchanges, radials, fusions and breaks. This increased level is similar to or slightly higher than the spontaneous level of chromosomal aberrations reported for FA-A and other FA cell lines [19,23]. Spontaneous chromosomal aberrations/breaks are characteristic of FA cells and thought to be an important determinant in the associated chromosomal instability associated with this disorder [19,23,24]. Based on the present results, it is possible to speculate that the spontaneous chromosomal aberrations/breaks that occur in FA cells could in part be due to their deficiency in α IISp. In normal cells in which α IISp has been knocked down, these chromosomal aberrations increased after exposure to MMC, just as they do in FA cells [19,23]. Though this increase was not as great as in FA cells, this may be attributable to the increased sensitivity of the α IISp siRNA transfected cells to MMC.

We have previously shown that reduced levels of α IISp in FA cells are not due to reduced expression of this protein but rather to decreased stability [18]. We have proposed that one or more of the FA proteins is important in maintaining the stability of α IISp in cells [17,18]. In support of this, we have shown that the FA protein, FANCG, binds directly to α IISp, specifically to the SH3 domain of α IISp [18]. This binding could be important for maintaining

the stability of α IISp [17]. Since FA genes are proposed to be involved in maintenance of genomic stability [8,23,24], it is possible that this may in part be through the potential role of one or more of the FA proteins in maintaining the stability of α IISp in the cell, which in turn is important for chromosomal stability.

We have also shown that α IISp plays a role in repair of DNA interstrand cross-links where it co-localizes in damage-induced nuclear foci with the cross-link repair protein, XPF, along with FANCA [5-7]. The present study shows that knockdown of α IISp in normal cells leads to decreased formation of α IISp and XPF nuclear foci after cross-link damage, even though levels of XPF in these cells are normal. This finding is similar to our demonstration that in FA-A cells, where levels of α IISp are similar to those in α IISp knocked-down cells, there is a reduction in damage-induced nuclear foci containing both α IISp and XPF, even though levels of XPF in these cells are normal [7]. The present results also show that the reduced formation of XPF foci in the damaged α IISp siRNA treated cells is not due to decreased levels of FANCA. Thus, in both FA-A cells and α IISp siRNA transfected normal cells, reduced formation of damage-induced XPF foci is due to a deficiency in α IISp in these cells. These results are in agreement with our model that α IISp acts as a scaffold in the nucleus to aid in the recruitment of repair proteins, such as XPF/ERCC1, to sites of damage, thus enhancing the efficiency of the repair process [6,7,17]. In FA cells [5,14,16], or normal cells in which α IISp has been knocked down, the reduced levels of α IISp lead to decreased levels of DNA repair.

Based on the present findings of development of chromosomal aberrations/breaks in cells in which α IISp has been knocked down, this model can now be extended to include a role for α IISp in maintaining genomic stability. Thus in FA cells, a deficiency in α IISp may not only lead to a defect in DNA repair but also contribute to the marked chromosomal instability associated with this disorder. Maintaining the stability of α IISp in the cell could thus be a critical factor in maintenance of chromosomal stability and, as our recent studies suggest, FA proteins may play an important role in this process [17].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the on line version, at.

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Fig. 1.

Knocking down of α IISp in normal lymphoblastoid and HeLa cells by siRNA. (A) Immunoblot analysis of normal lymphoblastoid cells 24 and 48 hr after transfection with 25 pmol of α IISp siRNA or nontarget (Nt) siRNA. Blots were probed with anti- α spectrin. Tubulin was used as a loading control. (B) Immunoblots were scanned and levels of α IISp quantitated. (C) Immunoblot analysis of HeLa cells 24 and 48 hr after transfection with 30 pmol of α IISp or Nt siRNA. (D) HeLa cell survival 48 hours after transfection with increasing concentrations of α IISp or Nt siRNA. (E) Immunoblot of effect in HeLa cells of increasing concentrations of α IISp siRNA on levels of α IISp 48 hours post transfection. FANCA was used as a loading control. (F) RT-PCR analysis of total RNA extracted from normal lymphoblastoid cells 48 hours after transfection with 25 pmole α IISp or Nt siRNA. Vertical lines represent $\pm$ SEM.

Fig. 2.

Cellular morphology of α IISp siRNA transfected normal lymphoblastoid cells compared to FA-A lymphoblastoid cells. (A) Microscopic examination of normal cells 48 hours after transfection with 25 pmoles α IISp or Nt siRNA. This is compared to FA-A cells in culture. (B) Western blot analysis of levels of α IISp in the Nt and α IISp siRNA transfected normal cells shown in A. (C) Western blot analysis of the levels of α IISp in the FA cells shown in A compared to normal cells.

Fig. 3.

Analysis of chromosomal aberrations in normal lymphoblastoid cells transfected with α IISp siRNA. (A) Metaphase spreads from normal cells were examined for chromosomal aberrations 48 hours after transfection with 25 pmoles of α IISp or nontarget siRNA. Arrows indicate interchromatid exchanges ($\rightarrow$), radials ($\Rightarrow$) and breaks ($\rightarrow$). (B) 100 metaphase spreads were scored for intrachromatid exchanges, radials/fusions and breaks. Chromatid interchange figures were converted into breaks, where one interchange was counted as one break, to give the total number of chromatid breaks. Vertical lines represent $\pm$ SEM.

Fig. 4.

Effects of mitomycin C on normal lymphoblastoid cells transfected with α IIISp siRNA. (A) Cell growth was examined in normal cells 24 hours after treatment with varying concentrations of MMC (48 hours post transfection with α IIISp or nontarget siRNA). (B) Normal cells were examined for α IIISp or XPF nuclear foci 16 hours treatment with 400 nM MMC (40 hours after transfection with α IIISp (+) or nontarget (-) siRNA) using indirect immunofluorescence and staining with anti- α -spectrin or anti-XPF. Vertical lines represent $\pm$ SEM.