Inhibition of the de-myelinating properties of Aicardi-Goutières Syndrome lymphocytes by cathepsin D silencing

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ABSTRACT

Molecular mechanisms relating interferon-alpha (IFN-alpha) to brain damage have recently been identified in a microarray analysis of cerebrospinal fluid lymphocytes from patients with Aicardi-Goutières Syndrome (AGS). These findings demonstrate that the inhibition of angiogenesis and the activation of neurotoxic lymphocytes are the major pathogenic mechanisms involved in the brain damage consequent to elevated interferon-alpha levels. Our previous study demonstrated that cathepsin D, a lysosomal aspartyl endopeptidase, is the primary mediator of the neurotoxicity exerted by AGS lymphocytes. Cathepsin D is a potent pro-apoptotic, neurotoxic, and demyelinating protease if it is not properly inhibited by the activities of leukocystatins. In central nervous system white matter, demyelination results from cathepsin over-expression when not balanced by the expression of its inhibitors. In the present study, we used RNA interference to inhibit cathepsin D expression in AGS lymphocytes with the aim of decreasing the neurotoxicity of these cells. Peripheral blood lymphocytes collected from an AGS patient were immortalized and co-cultured with astrocytes in the presence of interferon alpha with or without cathepsin D RNA interference probes. Cathepsin D expression was measured by qPCR, and neurotoxicity was evaluated by microscopy. RNA interference inhibited cathepsin D over-production by 2.6-fold ($P < 0.01$) in AGS lymphocytes cultured in the presence of interferon alpha. AGS lymphocytes treated using RNA interference exhibited a decreased ability to induce neurotoxicity in astrocytes. Such neurotoxicity results in the inhibition of astrocyte growth and the inhibition of the ability of astrocytes to construct web-like aggregates.

These results suggest a new strategy for repairing AGS lymphocytes in vitro by inhibiting their ability to induce astrocyte damage and leukodystrophy.

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1. Introduction

Aicardi-Goutières Syndrome (AGS) is a rare severe encephalopathy of mutational origin that is characterized by increased levels of interferon-alpha in the cerebrospinal fluid (CSF) and arises in infants during the first year of life. Molecular mechanisms relating interferon-alpha and brain damage have been recently identified in a microarray analysis of CSF AGS lymphocytes, demonstrating that the inhibition of angiogenesis and the activation of neurotoxic lymphocytes are the major pathogenic mechanisms involved. AGS lymphocytes are characterized by profound alterations in gene expression that primarily affect IFN-alpha-dependent lymphocyte activation and the inhibition of angiogenesis [1,2]. A remarkable level of inter-individual variation was observed in the gene expression profile of AGS lymphocytes depending on the specific AGS mutations present and the clinical course of the disease [3].

The activation of these pathogenic mechanisms results in the inhibition of neural cell growth, the insufficient development of brain vessels, de-myelination, and severe leukodystrophy. Cathepsin D was identified by qPCR as the primary mediator produced by

TREX1-mutated lymphocytes that is involved in the inhibition of neuroblastoma cell growth. These effects were enhanced in the presence of interferon alpha [4].

In the peripheral blood, the cathepsin B and D activity levels are altered in monocytes from systemic lupus erythematosus (SLE) patients, with the level of alteration dependent on the disease status [5]. This relationship indicates that the functional alteration of phagocytic cells is related to intracellular overload due to defects in peptide digestion [6,7]. In the central nervous system (CNS), cathepsin D expression is twofold higher in cerebrospinal fluid (CSF) AGS lymphocytes than in control lymphocytes [1]. Studies using RNA interference have confirmed that cathepsin D has a role in the activation of inflammation [5]. Cytoplasmic RNA overload results in cathepsin-dependent inflammation and the activation of caspase 1- and 3-dependent apoptosis in human macrophages [8]. This study indicates that apoptosis triggered by viral dsRNA is blocked by the inhibition of cathepsins, thus highlighting the importance of cathepsins in the innate immune response. It is notable that an intracellular overload of short RNAs with an endogenous origin occurs in AGS and plays a pathogenic role [9].

In CNS white matter, demyelination results from cathepsin overexpression when not balanced by the expression of their primary inhibitors, i.e., extracellular cystatins C and F. An unbalanced cathepsin/cystatin ratio plays a pivotal role in demyelination and leukodystrophy progression, which hamper the re-myelination processes, as demonstrated in a mouse model of multiple sclerosis [10]. An inverse relationship between cystatin expression and cathepsin expression in Aicardi-Goutières patients has been observed by analyzing gene expression in cerebrospinal lymphocytes using cDNA microarray technology [1].

Based on these past results, the goal of the study presented herein was to investigate the possibility of repairing AGS lymphocytes in vitro by suppressing cathepsin D expression through RNA interference. RNA interference (RNAi) is performed by the intracellular delivery of oligonucleotide probes that target specific messenger RNAs whose expression is then arrested.

The feasibility of repairing SLE lymphocytes in vitro and reimplanting them in vivo, thus attenuating the clinical phenotype, has been demonstrated. Murine SLE lymphocytes were submitted to gene transfer with a consensus sequence for anti-DNA IgG T-cell determinants, and this gene transfer delayed the development of lupus nephritis and suppressed the related hypergammaglobulinemia [11].

This approach was used to mitigate the ability of AGS lymphocytes to induce astrocyte cytotoxicity either under basal conditions or following activation by IFN-alpha. These ex-vivo experiments were used to evaluate the feasibility of attenuating cathepsin expression by RNA interference.

2. Materials and methods

2.1. Co-culture of lymphocytes and astrocytoma cells

Human U87 MG astrocytoma cells were obtained from the Cell Factory IST, Genoa, Italy. U87 MG cells were cultured in DMEM in the presence of 10% fetal calf serum and 1% glutamine at 37 °C and 5% CO_2. The cells were grown on sterilized culture dishes and were split every 2 days using 0.25% trypsin (Invitrogen, Life Technologies Europe BV Monza, Italy). For the RNA interference experiments, 5×10^5 cells/500 µl were seeded in 24-well plates 24 h prior to transfection. Lymphocytes were isolated from freshly collected whole blood by Histopaque®-1077 (Sigma–Aldrich, St. Louis, MO) gradient centrifugation and were placed in co-culture baskets (70,000 cells/basket) (Greiner Bio-One, Munich, Germany).

Interleukin 2 (4 µg/ml) and phytohemagglutinin (2 µg/ml) were added to the culture medium.

Cell lines derived from lymphocytes obtained from a healthy control (male, 6 years old) and an AGS patient (male, 6 years old) were comparatively examined. The AGS patient was identified as heterozygous for a double AGS1 TREX mutation (c.262 ins AG het + c.290 g > a R97H het) (courtesy of Professor Y. Crow, University of Manchester, UK). The enrolled subjects were treated in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of the IRCSS Mondino, University of Pavia, Italy. The Epstein–Barr (EBV) virus was used to convert the lymphocytes to continuously dividing, efficiently immortalized lymphoblastoid cell lines. The transforming virus was obtained from the lymphoblastoid marmoset cell line B95–8 (European Collection of Cell Cultures ECACC, Sigma–Aldrich St. Louis, MO USA). This line was established by infecting marmoset lymphocytes with EBV from a human patient with infectious mononucleosis. Cyclosporine A was used as an immunosuppressive drug to enhance the outgrowth of EBV-infected lymphocytes [12]. Peripheral blood mononuclear cells were resuspended in RPMI-1640 growth medium (GE Healthcare Life Sciences United Kingdom) supplemented with 20% fetal bovine serum (GE Healthcare Life Sciences United Kingdom), 1% 200 mM L-glutamine (GE Healthcare Life Sciences United Kingdom), 1% penicillin streptomycin (GE Healthcare Life Sciences) and 5 µg/µl of CS-A (Sigma–Aldrich, St. Louis, MO USA) and the same volume of virus-containing supernatant from B95–8 cells. After 1 week in culture, half of the growth medium was removed and replaced with fresh medium containing cyclosporine A. After 2–3 weeks in culture, small clumps became visible. The cell lines were then stored at −180C in liquid nitrogen until use.

U87 MG (70,000 cells) and lymphocytes (100,000 cells) from the AGS patient and the control were co-cultured with interferon-alpha at 2500 U/ml (Sigma–Aldrich, St. Louis, MO USA) for 24 h.

2.2. RNA interference-mediated inhibition of cathepsin D in AGS and control lymphocytes

The post-transcriptional silencing of the cathepsin D gene was induced by double-stranded RNA interference using a commercially available kit (Origene Trilencer-27 siRNA knockdown duplexes, Medical Center Dr., Suite 200, Rockville, MD 20850). Three RNA interference oligonucleotide probes specific for cathepsin D were used. Each silencing RNA oligonucleotide (siRNA) was added to 100 µl RNase-free Duplex Buffer (20 µM). The mixture was heated at 94 °C for 2 min and then cooled to room temperature. The mixture was then added to 10 µl of Opti-MEM, 0.3 µl of siRNA (5 µM) and siTran transfection reagent (Origene). After 24 h, the lymphocytes were pelleted at $800 \times g$ for 5 min at 4 °C, and the level of cathepsin D knockdown was analyzed by qPCR.

The sequences of the three siRNAs were designed and tested using a bioinformatic approach with an ad hoc program (Beacon Designer 7.51, Premier Biosoft International, Palo Alto, CA, USA) considering the following parameters: (a) the binding affinity for the cathepsin D messenger RNA; (b) the specificity toward the cathepsin D messenger RNA; and (c) the absence of non-specific cross-reactivity with other messenger RNAs (BLAST analysis). The three siRNA sequences used were as follows:

A) SR301070A: AGACUCCAAGUAUUACAAGGGUUCT
B) SR301070B: GCUCAAGAACUACAUGGACGCCCAG
C) SR301070C: CGCCAGCACAGAAACAGAGGAGAGT

Sham control samples were submitted to the same procedure in the absence of siRNA probes.

2.3. Transfection using the 4D-Nucleofector X Unit Amaxa

To increase the efficacy of the siRNA transfection, a multi-well high-efficiency electroporator was used (4D-Nucleofector™ X Unit Amaxa, Lonza Cologne AG 50829 Cologne, Germany). A commercially available kit was used for lymphocyte electroporation (V4XP-3032 Primary Cell 4D-Nucleofector X Kit S (32 RCT), Amaxa) with a cell density of 1 million cells/well. To verify the transfection efficiency, siRNA probes were labeled with green fluorescent protein (GFP) (GFPT2 human Origene), and labeled cells were quantified using a Burker chamber. This procedure allows the application of an electric field with a low intensity, which alters the permeability of the cell membrane, thus facilitating the entrance of oligonucleotide probes. The use of a multi-well electroporator allowed the simultaneous analysis of 96 multiple experimental conditions, thus allowing the rapid identification of the best conditions for the transfection. It is crucial to adequately modulate the intensity and duration of the electric field to maximize the penetration of the molecular probe and minimize damage to the cells.

The cell viability after transfection was determined using the MTT assay.

2.4. MTT assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. MTT (Sigma Chemical Company (St. Louis, MO, USA) solution (5 mg/ml of MTT in PBS) was added to each well for 3 h. A cell-free-medium blank was included in each experiment. The absorbance of each well was measured using a microplate reader (Multiskan FC M Medical, Milano) at a wavelength of 570 nm. The absorbance of the control was considered indicative of 100% viability.

2.5. Quantitative PCR

The efficiency of the siRNA-mediated inhibition of cathepsin D expression was evaluated by real-time quantitative PCR (qPCR). In this assay, control lymphocytes were compared with AGS lymphocytes treated with 2500 U/ml IFN alpha for 24 h. Total RNA was extracted using TRIzol (Invitrogen Corp., Carlsbad, CA, USA), and the purity and integrity of the RNA were evaluated using fiber optic spectrophotometry (NanoDrop, Thermo Fisher Scientific, Wilmington, DE, USA) and capillary electrophoresis (Bioanalyzer, Agilent Technologies, Inc., Santa Clara, CA, USA).

Total RNA (50 ng) was added to 0.5 μg oligo(dT), 10 mM dNTP mix (1 μl), and water to a total volume of 12 μl, and the mixture was incubated at 65°C for 5 min. Subsequently, 5× first-strand buffer (4 μl), 0.1 M DTT (2 μl), RNase OUT (1 μl), and DNA reverse transcriptase superscript II (1 μl) (Invitrogen, United Kingdom) were added, and the mixture was incubated for 60 min at 42°C, followed by 15 min at 70°C to stop the reaction. Finally, 2 μl of the obtained cDNA was added to a 200 μl vessel containing 10× PCR buffer (5 μl), 50 mM $MgCl_2$ (2 μl), 100 mM dNTP mix (0.4 μl), 10 μM primer A (1 μl), 10 μM primer S (1 μl), Platinum® Taq DNA polymerase (0.5 μl) (Invitrogen) and 10 μM specific molecular beacon (2 μl) (Tib MolBiol GmbH-Eresburgstrasse 22–23 D-12103 Berlin). The qPCR reaction was carried out in a rotating thermocycler (Rotor-Gene 3000, Corbett Research, Mortlake, Australia) using the following program: 95C for 2 min; 40 cycles at 94°C for 45 s, 24°C for 30 s, and 72°C for 30 s. A second parallel qPCR reaction was performed using primers and probes specific for the housekeeping gene GAPDH, and the level of GAPDH was used to normalize the cathepsin D expression data among the tested samples. Statistical analyses were executed using StatView software (Abacus Concept, Berkeley, CA, USA).

3. Results

Lymphocytes are quite resistant to cell transfection. However, our results provide evidence that multiwell electroporation is an adequate approach for transfecting these cells with siRNA probes. Fluorescence microscopy (Fig. 1) indicated that the human lymphocytes were effectively transfected by the GFP-labeled siRNA probes. The transfection efficiency for control lymphocytes was 28.0%. By contrast, the transfection efficiency was only 1.1% when the lymphocytes were transfected without electroporation using only Lipofectamine-based transfection reagents.

Using electroporation, the transfection efficiency of AGS lymphocytes was 27.2%, i.e., equal to that obtained in normal lymphocytes. No signs of cell damage were observed either by microscopy (Fig. 1, upper panel) or the MTT assay (100.0% vs 98.7%, control vs transfected cells).siRNA transfer resulted in the silencing of cathepsin D expression. As evaluated by qPCR (Fig. 2, upper panel, blue lines), the expression of cathepsin D mRNA was 2.0-fold higher in AGS lymphocytes in the absence of IFN-alpha compared to control healthy lymphocytes. IFN-alpha stimulated cathepsin D expression both in control (1.4-fold) and AGS lymphocytes (2.6-fold). These data indicate that AGS lymphocytes have a higher
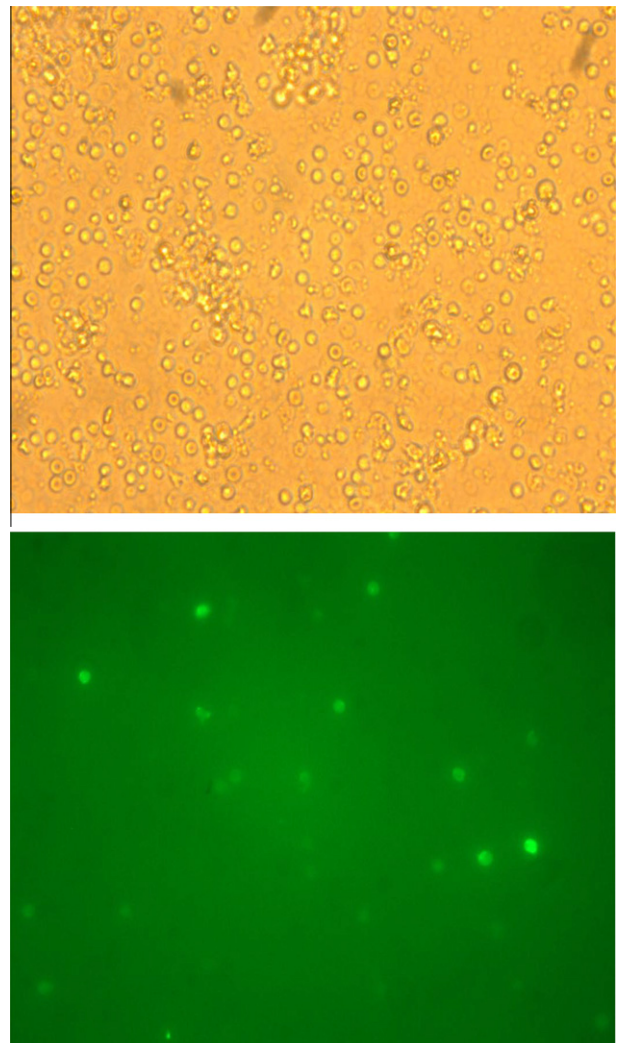
Fig. 1. Immortalized lymphocytes undergoing GFP-siRNA transfection by electroporation. No change in cell morphology was observed after transfection (upper panel). GFP-siRNA penetrated 28% of lymphocytes, as observed by fluorescence microscopy (lower panel).
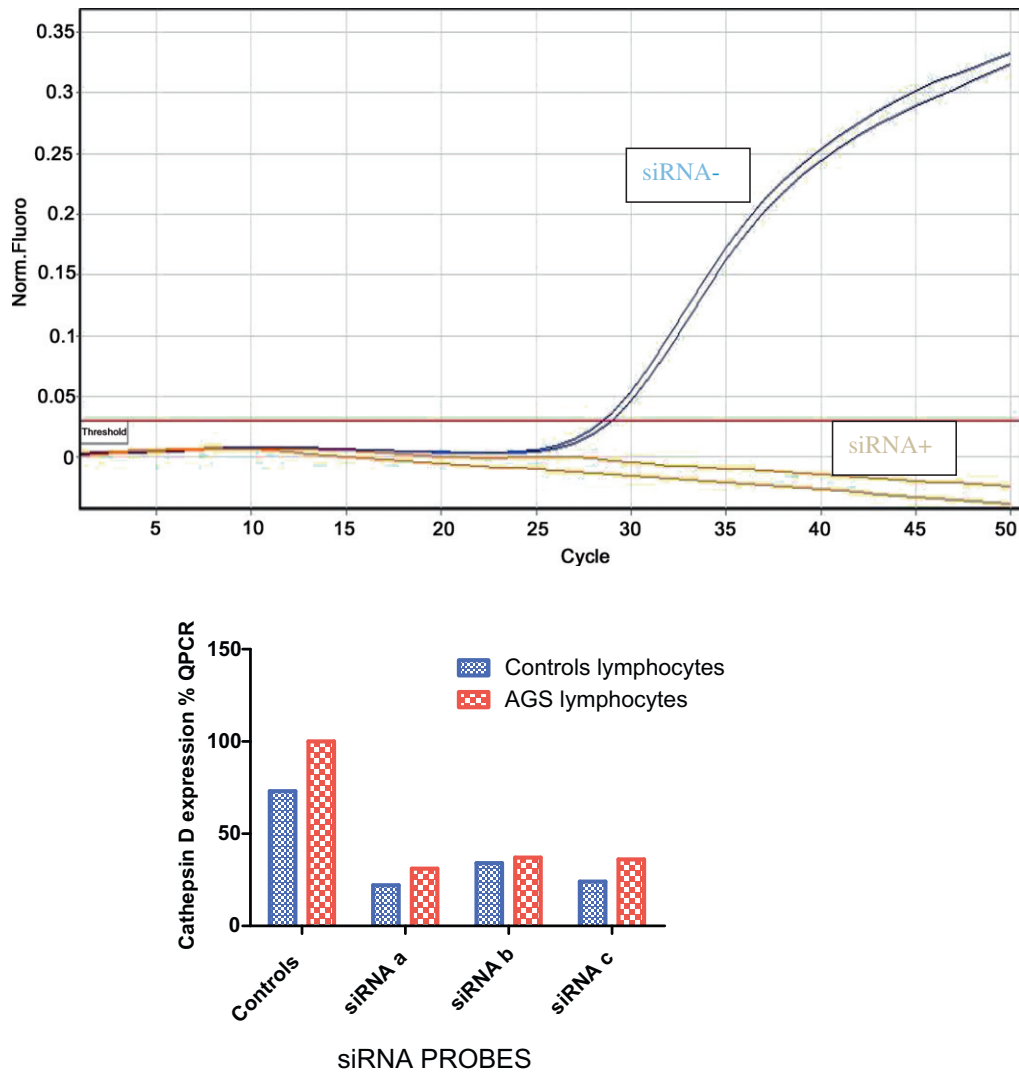
Fig. 2. Upper panel: Analysis of cathepsin D expression by qPCR in the blood lymphocytes of AGS patients in the presence of IFN-alpha before (blue lines) and after siRNA transfection (yellow lines). The red line indicates the threshold for qPCR positivity. Lower panel: Silencing efficiency obtained for each single siRNA probe (**$P < 0.01$, transfected vs non-transfected). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

basal expression level of cathepsin D than control lymphocytes in the presence of IFN-alpha and are more susceptible than control lymphocytes to IFN-alpha stimulation. At 24 h after transfection, the siRNA probes resulted in significant ($P < 0.01$) decreases in cathepsin D expression in both AGS and control lymphocytes. Three siRNA probes (A, B, and C) were evaluated, and the silencing efficiency was not significantly different among them. The greatest level of inhibition was obtained using siRNA probe A, which reduced the expression of cathepsin D from 100% to 31% in AGS lymphocytes stimulated with IFN-alpha (Fig. 2, lower panel).
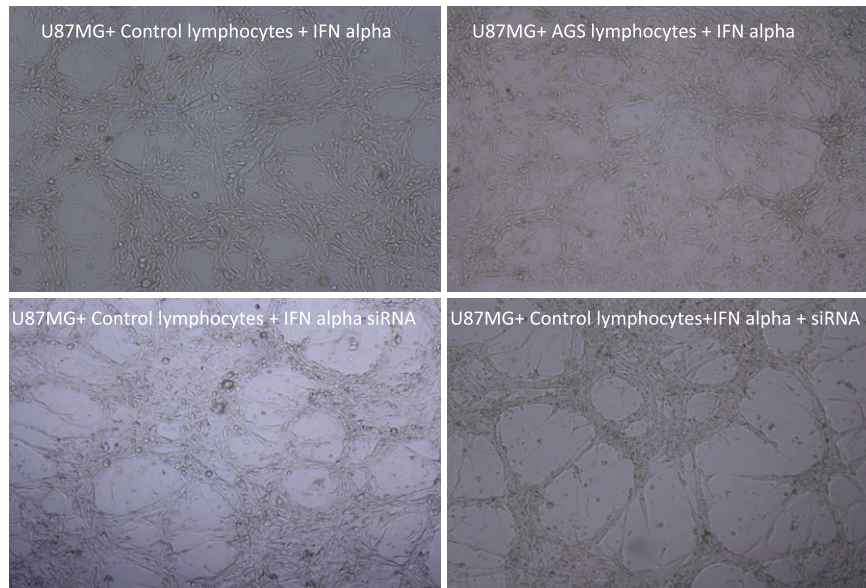
This modulation of cathepsin D expression resulted in functional changes in AGS lymphocytes, and their neural cytotoxicity was evaluated in co-culture experiments. As shown in Fig. 3 (upper panels), compared with the control lymphocytes, AGS lymphocytes stimulated with IFN-alpha remarkably reduced the ability of co-cultured astrocytes to form intercellular budges and web-like aggregates. Furthermore, when co-cultured with AGS lymphocytes, astrocytes were thinner, of even morphology, and spheroidal, features that indicate poor cell health. Conversely, when co-cultured with AGS lymphocytes transfected with siRNA to inhibit cathepsin D expression, the astrocytes did not show any signs of poor cell health, and their morphology and growth were comparable to

those observed in the co-culture with control lymphocytes (Fig. 3, lower panel).

4. Discussion

The results obtained in this study indicate that RNA interference can be used to inhibit the over-production of cathepsin D that occurs in AGS lymphocytes in the presence of interferon alpha. This inhibition is reflected by the expression of this gene in AGS lymphocytes relative to the expression level in the control lymphocytes. The high transfection efficiency obtained prompted us to hypothesize that it will be feasible to treat AGS lymphocytes in vitro and then reintroduce them in vivo to mitigate the pathogenic mechanisms of AGS. Thus, this research may allow the development of new strategies for the treatment and secondary prevention of AGS. The imbalance between myelin-directed extracellular proteases and their specific inhibitors is a driving pathogenic mechanism of the leukodystrophy that occurs in AGS patients. This imbalance characterizes young patients affected by AGS, a genetic disease that is associated by definition with severe leukodystrophy and microcephaly occurring during the first year

Fig. 3. Decrease in the neurotoxicity of AGS lymphocytes in the presence of IFN-alpha due to the inhibition of cathepsin D expression after transfection of the cells with siRNA probes.

of life [13]. The qPCR analysis demonstrated that cathepsin D expression was remarkably increased in the AGS lymphocytes collected from the cerebrospinal fluid relative to the expression level in lymphocytes from an age-matched control. Furthermore, cathepsin D expression was induced by IFN-alpha, whose presence in the cerebrospinal fluid is a hallmark of AGS.

Cathepsin D inhibition could be the basis of a therapeutic approach because of the multiple pathophysiological roles of this protease, which plays a major role in inflammation, apoptosis, and autophagy.

As part of the inflammatory response, cathepsin D is increased in the plasma soon after severe tissue injury, whose expression is indicative of complement activation [14]. Macrophage death triggered by the presence of viral double-stranded RNA in the cytoplasm can be blocked by the presence of cathepsin, by increasing cathepsin B expression in the macrophages, and by reducing cystatin-cathepsin interactions [15]. Inappropriate immune activation typically occurs in AGS patients, in whom endogenous RNA/DNA cannot be removed due to mutations that silence genes encoding RNases [16–18]. Intracellular overload with short RNAs/DNAs activates Toll-like receptors, and the Pol-III pathway [19], thus triggering interferon-alpha production, lymphocytosis, angiogenesis inhibition, and leukodystrophy [1] and blocking the microRNA machinery [9]. In AGS patients, the overproduction of IFN-alpha activates the intracellular mediator mTOR, resulting in the production of calcineurin, and subsequently leukodystrophy. This activation of mTOR is in line with the established role of this protein in neurodegenerative disease [20]. Cystatins C and F are, on their own, inhibitors of both mTOR and cathepsin. The interactions between cystatins and the aspartic proteinase cathepsin D at pH 3.5 result in the inactivation of cathepsin D accompanied by peptide bond cleavage at several sites, preferentially those involving hydrophobic amino acid residues [21]. Accordingly, cystatins are able to arrest demyelination in homozygous knockout mice (plp$^{tg/-}$ mice), which exhibit demyelination similar to that observed in individuals with multiple sclerosis [22]. Cystatin expression in the CSF lymphocytes of AGS patients is inversely related to IFN-alpha and cathepsin D expression. The age-related increase in cystatin expression in AGS patients is associated with the arrest of the progression of leukodystrophy [1].

Cathepsins were originally thought to play a central role in protein degradation within the lysosomal compartment. However, the suppression of cathepsin D expression using a cathepsin D-specific siRNA failed to protect cells from oxidative stress-induced cell death [23].

Cathepsin D also plays a role as an extracellular cell death activator. Activated lymphocytes release cathepsins into the extracellular space, where these proteins can reach their target cells. By targeting this mechanism, pharmacological inhibitors of cathepsin D and the siRNA-mediated suppression of this protease reduce the level of cell death in Mv1 Lu type II alveolar cells [24].

The high level of cathepsin D expression in cells is related to the activation of autophagic processes [25]. The over-expression of wild-type cathepsin D, but not a catalytically inactive mutant of cathepsin D (D295 N), inhibited H_2O_2-induced cell death in HeLa cells. Interestingly, a high expression level of cathepsin D in HeLa cells significantly activated autophagy, as observed by an increase in the number of acidic autophagic vacuoles, LC3-II formation, and GFP-LC3 puncta.

All these cathepsin D functions are relevant to the development of neurodegenerative diseases. Cathepsin D has been implicated in the proteolysis of ApoE [26]. In parallel, hypermyelination and altered myelin integrity are observed in *Cathepsin d$^{-/-}$* transgenic mice. Structural alterations in myelin have been shown to be associated with the accumulation of cholesteryl esters and abnormal fatty acyl and alkenyl chains in the brains of *Cathepsin d$^{-/-}$* mice, indicating that there is pronounced degradation of myelin in these brains. Cathepsin D contributes to microglia neurotoxicity in vitro and to inflammation-mediated neurodegeneration in vivo [27]. Additionally, the knockdown of cathepsin D expression in microglia using short hairpin RNA diminished the level of neurotoxicity in a co-culture of microglia and neuroblastoma cells [27]. Indeed, cathepsin D plays a central role in microglia neurotoxicity, and it is a potential biomarker and drug target for the diagnosis and treatment, respectively, of neurodegenerative diseases associated with excessive microglial activation and subsequent neurotoxic inflammation. These mechanisms explain why the inhibition of cathepsin D by siRNA results in a decrease in the cytotoxic effects of AGS lymphocytes on astrocytes in co-culture.

In AGS patients, nucleic acids accumulate, leading to the erroneous activation of the innate immune system. Based on observations made in TREX1-deficient mice and cells [28,29], it has been proposed that endogenous retro-elements represent a major species which, in the absence of TREX1, accumulate in the cytosol and trigger innate immune activation.

The in vitro data presented herein indicate that this innate immune activation could be attenuated, inhibiting cathepsin D expression by RNAi in lymphocytes activated by IFN-alpha. This approach was able to attenuate the production of this demyelinating protease, which triggers the pathogenic mechanisms of neurotoxicity in AGS lymphocytes. The high transfection efficiency obtained by electroporation makes plausible a future in vivo approach based on the collection of lymphocytes from AGS patients, the repair of these cells by siRNA-mediated inhibition of cathepsin D over-expression, and their reintroduction into AGS patients. Similar procedures have already been proposed in experimental animal models for the treatment of SLE and are already clinically used for immunotherapy [30].

Acknowledgments

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