The Friedreich’s ataxia mutation confers cellular sensitivity to oxidant stress which is rescued by chelators of iron and calcium and inhibitors of apoptosis

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Expansions of an intronic GAA repeat reduce the expression of frataxin and cause Friedreich’s ataxia (FRDA), an autosomal recessive neurodegenerative disease. Frataxin is a mitochondrial protein, and disruption of a frataxin homolog in yeast results in increased sensitivity to oxidant stress, increased mitochondrial iron and respiration deficiency. These previous data support the hypothesis that FRDA is a disease of mitochondrial oxidative stress, a hypothesis we have tested in cultured cells from FRDA patients. FRDA fibroblasts were hypersensitive to iron stress and significantly more sensitive to hydrogen peroxide than controls. The iron chelator deferoxamine rescued FRDA fibroblasts more than controls from oxidant-induced death, consistent with a role for iron in the differential kinetics of death; however, mean mitochondrial iron content in FRDA fibroblasts was increased by only 40%. Treatment of cells with the intracellular Ca²⁺ chelator BAPTA-AM rescued both FRDA fibroblasts and controls from oxidant-induced death. Treatment with apoptosis inhibitors rescued FRDA but not control fibroblasts from oxidant stress, and staurosporine-induced caspase 3 activity was higher in FRDA fibroblasts, consistent with the possibility that an apoptotic step upstream of caspase 3 is activated in FRDA fibroblasts. These results demonstrate that FRDA fibroblasts are sensitive to oxidant stress, and may be a useful model in which to elucidate the FRDA mechanism and therapeutic strategies.

INTRODUCTION

Friedreich’s ataxia (FRDA), the most common hereditary ataxia, is an autosomal recessive disease characterized by unsteady gait, muscle weakness of the legs, neuropathy and cardiomyopathy (¹). Degeneration of the spinal cord, mainly the posterior columns and spinocerebellar tracts, occurs in FRDA patients. The cervical region is most severely damaged along with loss of large cells in the dorsal root ganglia, followed by loss of large myelinated axons in the peripheral nerve (²).

The genetic defect observed most frequently in FRDA patients is a GAA repeat expansion in the first intron of the gene encoding frataxin, a protein of unknown function (³). The GAA repeat expansion appears to inhibit both gene transcription and replication (⁴). While normal individuals possess 8–30 GAA repeats, FRDA patients have from 160 to >1200 copies of the GAA repeat (³,⁵). The genome of Saccharomyces cerevisiae contains a frataxin homolog, YFH1 (yeast frataxin homolog), knockout of which causes increased sensitivity to oxidative stress induced by hydrogen peroxide (H₂O₂), iron and copper, and an increased level of mitochondrial iron (⁶,⁷). Depletion of mtDNA and a decrease in respiration were observed in ΔYFH1 cells (⁷,⁸). In human cells, frataxin has been localized to mitochondria (⁹), and is highly expressed in heart, muscle, liver and pancreas (³,⁹). Interestingly, decreases in the activity of the mitochondrial iron–sulfur-containing proteins aconitase and respiratory chain

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complexes I, II and III have been observed in heart biopsies from a limited number of FRDA patients (10), which would suggest a pathogenic role for oxidant stress, as iron–sulfur proteins are exquisitely sensitive to the accumulation of reactive oxygen species.

Studies reported here address whether human FRDA fibroblasts may be a reasonable model system in which to study the cellular effects of GAA expansion (i.e. whether the mutation confers a predisposition to cell death), and also to test in this model system potential therapeutic agents that would be expected to inhibit death induced by oxidant stress on mechanistic grounds.

RESULTS

Levels of frataxin mRNA levels are reduced in FRDA fibroblasts

Frataxin mRNA levels were measured by RT–PCR and normalized to those of an unrelated mitochondrial protein, β-MTP, the β-subunit of mitochondrial trifunctional protein. The level of frataxin expression was 33.6% of the control value (see Materials and Methods).

FRDA fibroblasts are more sensitive to H₂O₂ and iron than controls

The sensitivity of five fibroblastoid FRDA cell lines and five control lines to H₂O₂ was examined. FRDA cells exhibited significantly greater sensitivity than controls to H₂O₂ at doses of 50 and 100 µM H₂O₂ (Fig. 1a). FRDA and control cells exhibited 81.7 ± 6.8 (95% CI) versus 93.2 ± 1.0% viability at 50 µM H₂O₂, and 51.6 ± 3.2% and 86.4 ± 6.2% at 100 µM H₂O₂, respectively.

FRDA fibroblasts were hypersensitive to iron, an element known to induce oxidative stress, relative to controls (Fig. 1b). Control cells maintained nearly 100% viability at up to 5 mM FeCl₃, while only 50% of the FRDA fibroblasts were viable at 1 mM FeCl₃.

An iron chelator rescues FRDA fibroblasts preferentially from death

Deferoxamine mesylate is a specific iron chelator used in clinical practice (11). FRDA fibroblasts (Fig. 2b) treated with deferoxamine were rescued from H₂O₂-induced death to a greater extent than controls (Fig. 2a).

One possible cause of increased sensitivity to oxidant stress in the human FRDA fibroblasts is an increased concentration of mitochondrial iron, as has been observed in the yeast knockout of the frataxin homolog (6,7). We did observe a small (40%) increase in mean concentration of mitochondrial iron in FRDA fibroblasts: 8.93 versus 6.36 nmol/mg in controls; however, this difference was not statistically significant (Fig. 3a). Other experiments with three FRDA and three control lymphoblast lines gave similar results, i.e. a 30% increase in mean mitochondrial iron content, but the difference in means was not significant at the 95% confidence level (data not shown).

Figure 1. (a) Sensitivity of FRDA fibroblasts to oxidative stress. Cells were exposed to increasing concentrations of H₂O₂ for 6 h, after which time viability was determined by the trypan blue exclusion assay. Means of 28 independent experiments are shown and bars represent two standard errors of the mean, which in normally distributed data are considered to represent 95% confidence intervals. Filled squares and circles represent the mean survival of five control and five FRDA fibroblast lines, respectively. Significance levels at each individual dose are represented by asterisks: *P < 0.05 and **P < 0.005. (b) Hypersensitivity of FRDA fibroblasts to Fe³⁺. Five control and five FRDA fibroblast cell lines were exposed to increasing concentrations of iron. Filled squares, FRDA. Significance levels at each individual dose are represented by asterisks: **P < 0.005. Means of three independent experiments and error bars representing two standard errors of the mean are shown.

Pyruvate and uridine supplementation preferentially boosts growth of FRDA fibroblasts

Supplementation of tissue culture medium with uridine and pyruvate rescues growth defects resulting from mitochondrial dysfunction, presumably because the main function of mitochondria in a high-glucose tissue culture environment is to provide pyrimidines for nucleic acid synthesis (12,13). If frataxin expansions cause mitochondrial dysfunction, then one would expect that supplementation of tissue culture medium with
Protection of cells from H$_2$O$_2$-induced death by the iron chelator deferoxamine mesylate (DF). Cells were exposed to 250 µM DF for 18 h prior to the addition of H$_2$O$_2$. The mean survival of (a) five control and (b) five FRDA fibroblasts is shown. Significance levels at each individual dose are represented by asterisks: **P < 0.005. Averages of 28 and three independent experiments are shown for cells with or without deferoxamine, respectively, and bars represent two standard errors of the mean.

Depletion of intracellular calcium ion rescues FRDA fibroblasts and controls from oxidant-induced death

Although the complete mechanism by which oxidant stress kills cells is not completely understood, oxidant stress often causes a rise in intracellular Ca$^{2+}$, which may be required for cell death (14,15). Treatment of cells with an intracellular Ca$^{2+}$ chelator, BAPTA-AM, provided significant rescue of both control and FRDA fibroblasts from oxidant-induced death (Fig. 4), and depletion of Ca$^{2+}$ from cell media also provided significant rescue (data not shown).

We previously have shown that cyclosporin A (CsA) can protect cells with mitochondrial mutations from oxidant-induced death (14), presumably by blocking the mitochondrial permeability transition (MPT). To investigate this possibility of protection in FRDA fibroblasts, cells were pre-treated with 1 µM CsA for 30 min before addition of H$_2$O$_2$, and no protection was observed in FRDA nor control fibroblasts (data not shown).
Figure 4. Protection of FRDA and control fibroblasts from death induced by oxidant stress by the intracellular Ca\(^{2+}\) chelator BAPTA-AM. Survival of (a) five control cell lines and (b) five FRDA fibroblasts. \(\bullet\) cells; \(\circ\) cells + 10 \(\mu\)M BAPTA-AM. Significance levels at individual doses are represented by asterisks: **\(P < 0.005\). Averages from three and 28 independent experiments are shown for cells with or without BAPTA-AM, respectively, with bars representing 95% CI.

Apoptosis inhibitors preferentially rescue FRDA fibroblasts from death

Recently, mitochondrial disruption has been shown to trigger the apoptotic cascade directly (16–20). If mitochondrial stress in mutant FRDA fibroblasts resulted in activation of the apoptotic cascade, then one might predict that inhibitors of apoptosis should rescue FRDA fibroblasts preferentially from death. The apoptosis inhibitor z-VAD.fmkk conferred significant rescue on FRDA fibroblasts, but not controls (Fig. 5a and b). Other inhibitors of apoptosis, YVAD-CHO and DEVD-CHO, provided similar extents of rescue (data not shown). For example, at 250 \(\mu\)M \(\text{H}_2\text{O}_2\), the addition of z-VAD.fmkk increased the viability of FRDA fibroblasts from 21.0 ± 3.4 to 66.6 ± 8.8%, whereas the viability of control cells at this dose was not significantly different. z-VAD.fmkk did not provide significant protection of control cells from death, consistent with the view that caspase activity is not required for death of control cells. In fact, at the 100 \(\mu\)M dose of \(\text{H}_2\text{O}_2\), z-VAD.fmkk appeared to accelerate death slightly, an unexpected result.

DISCUSSION

The results presented above demonstrate that the GAA expansion in frataxin confers \(\text{H}_2\text{O}_2\) sensitivity and iron hypersensitivity on human FRDA fibroblasts. The kinetics of FRDA fibroblast death were dependent on the concentration of \(\text{H}_2\text{O}_2\) and iron, consistent with an oxidative stress hypothesis for FRDA. Iron is a crucial reagent in the Fenton reaction, as it can react with mitochondrially produced superoxide anion (\(\cdot\text{O}_2^-\)) to produce toxic free radicals such as the hydroxyl radical (\(\cdot\text{OH}^-\)). Thus, consistent with the knockout yeast model \(\Delta\text{YFH1}\), the results presented here support a pathogenic role for oxidative stress in FRDA. The iron chelator deferoxamine specifically rescued FRDA and, to a lesser extent, control cells from death, consistent with a death mechanism dependent on Fenton chemistry. Although mean iron levels were slightly higher in FRDA mitochondria than in controls, the difference was not statistically significant in the two fibroblast (Fig. 3a) and three lymphoblast (data not shown) lines examined. The small difference in iron concentration may be the consequence of residual frataxin expression in FRDA fibroblasts, in contrast to the situation in knockout yeast with zero expression of \(\text{YFH1}\). An alternative explanation is that in comparison with controls, FRDA fibroblasts either may experience intrinsically higher endogenous levels of oxidant stress or contain an oxidant-sensitive target, which would explain the sensitivity of FRDA fibroblasts to oxidants in the absence of a large difference in mitochondrial iron accumulation.

Increases in concentration of the intracellular Ca\(^{2+}\) ion are known to follow oxidant stress in many cell types (15). Chelation of intracellular Ca\(^{2+}\) rescued both FRDA and control fibroblasts from death, demonstrating a requirement for Ca\(^{2+}\) for oxidant-induced death, but not necessarily any difference in this Ca\(^{2+}\)-dependent step in FRDA versus control fibroblasts. In contrast to our earlier results in osteosarcoma cells bearing pathogenic mitochondrial DNA mutations in which CsA provided preferential protection from death induced by oxidant stress, presumably by inhibiting the MPT (14), we observed no protection by CsA of FRDA or control fibroblasts (data not shown). The implication is that oxidative stress is not killing FRDA fibroblasts by induction of the MPT.

Presumably a common endpoint of multiple toxic stimuli to cells is activation of the apoptotic machinery, which recently has been shown to be regulated at least partially at the mitochondrial level. If the FRDA mutation conferred excess activation of the apoptotic pathway, one would predict a greater sensitivity to agents which induce cell death (i.e. Fig. 1), and also a greater degree of protection from cell death by apoptosis inhibitors. The
Figure 5. (a and b) Protection of fibroblasts from H$_2$O$_2$-induced death by z-VAD.fmk. Cells were treated with H$_2$O$_2$ and z-VAD.fmk for 6 h and viability was assessed by the trypan blue exclusion assay. ■, cells; ○, cells + 50 µM z-VAD.fmk. Averages of three or 28 independent experiments are shown for cells treated with or without z-VAD.fmk, respectively. Significance levels at individual doses are represented by asterisks: *$P < 0.05$, **$P < 0.005$. (c) Induction of caspase 3 activity by staurosporine in FRDA fibroblasts versus controls. Specific activities for caspase 3 were 1.50 ± 0.24 (2x SEM) and 0.96 ± 0.19 pmol of p-nitroaniline/min/mg. Averages from 6–8 independent experiments are shown, with bars representing two standard errors of the mean.

staurosporine-induced caspase 3 activity was significantly higher in FRDA fibroblasts than in controls, and three known biochemical inhibitors of the apoptosis machinery provided significant protection of FRDA but not of control fibroblasts from death. These findings are consistent with the idea that the apoptotic pathway is activated preferentially in FRDA fibroblasts.

Altogether, our data suggest that FRDA may be the latest addition to the increasing list of neurodegenerative disorders caused by oxidative stress (21,22). The results indicate that expansions of GAA in the frataxin gene result in increased susceptibility of cells to oxidant-induced death, and thus the cells potentially could be used as a model to study the mechanism of death acceleration, which may relate to the FRDA disease process. In addition, the results demonstrate rescue from death by chelators of iron and calcium and by apoptosis inhibitors in the model system, which may suggest that antioxidant, chelation and anti-apoptotic therapy potentially could be considered for this devastating neurodegenerative disease.

MATERIALS AND METHODS

Reagents

H$_2$O$_2$ was purchased from Fisher (Pittsburgh, PA), 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetrakis(ace-toxymethyl)ester (BAPTA-AM) and deferoxamine mesylate were purchased from Sigma (St Louis, MO), z-VAD.fmk was purchased from Enzyme Systems (Livermore, CA), YVAD-CHO was purchased from Calbiochem (Cambridge, MA) and DEVD-CHO was purchased from Biomol (Plymouth Meeting, PA).

Patients, DNA analysis and cell culture conditions

All five FRDA fibroblast cell lines used in this study were from patients diagnosed with classical Friedreich’s ataxia. Mean passage numbers of FRDA and control fibroblasts were 15 and 13, respectively. Analysis of the GAA repeat expansion was performed by PCR as described (23) and the following allele sizes were determined: FRDA 13 (743/1046); FRDA 53 (658/1243); FRDA 209 (613/910); FRDA 1035 (365/742); FRDA 1037 (753/820); control 66LS (10/10); control 269 (8/11); control 305 (8/10); and controls GM00023 and GM00024 (NIGMS Human Genetic Mutant Cell Repository) (not determined). Expression of frataxin mRNA was measured by RT–PCR as described below. Mean expression of FRDA fibroblasts was 33.6% of the control mean. Fibroblasts were grown in minimal essential medium (MEM; Life Technologies, Gaithersburg, MD), supplemented with 8 mM glutamine, 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 10 µg/ml insulin, 10 ng/ml epidermal growth factor (EGF), 50 ng/ml basic growth factor (bFGF), and gentamicin.

Frataxin expression analysis

RT–PCR analysis of frataxin expression was performed in the five FRDA fibroblast cell lines as well as in five control cell lines. Poly(A)$^+$ mRNA was prepared from 4.5 x 10$^6$ cells using the FastTrack kit (Invitrogen, Milan, Italy) following the manufacturer’s instructions. cDNA was synthesized from 1 µg of poly(A)$^+$ mRNA using the Expand reverse transcriptase (Boehringer, Monza, Italy) and the ‘lock-docking’ protocol as previously described (24). A frataxin RT–PCR fragment of 480 bp was amplified using the sense oligonucleotide primer FA1/2-S
Cell viability was determined as described (14). Briefly, 1 × 10^5 cells were grown in MEM supplemented with 8 mM glutamine and 10% FBS 24 h prior to use. Cells were given fresh media (MEM, 8 mM glutamine and 10% FBS) before the addition of H_2O_2. The following reagents, with their final concentrations, were added to the cells before H_2O_2: 10 µM BAPTA-AM [in dimethyl sulfoxide (DMSO)], 50 µM z-VAD.fmk (in DMSO), 100 µM YYV-CHO (in water) and 100 µM DEVD-CHO (in water). Deteroxamine mesylate (250 µM final concentration, in water) was added to cells 18 h prior to H_2O_2. Six hours later, cells were removed by trypsin-EDTA and resuspended in phosphate-buffered saline. Viability was determined by the trypan blue exclusion assay. In the case of BAPTA and z-VAD.fmk, vehicle controls with DMSO were performed. Student’s t-tests were carried out to determine the significance values for both control and FRDA fibroblasts at individual doses.

Measurement of mitochondrial iron
Mitochondria were isolated from fibroblasts using the method of Rickwood et al. (25). Mitochondrial iron was measured by atomic absorption spectroscopy as described (26).

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