Received: 2017.02.20

Accepted: 2017.07.19

Published: 2018.02.27

Treatment of Donor Rat Hearts Prior to Transplantation with FLIP (FADD-Like Interleukin Beta-Converting Enzyme (FLICE)-Like Inhibitory Protein) in Cardioplegic Solution Decreased Apoptosis at Thirty Minutes Post-transplantation and Decreased Total Tyrosine Phosphorylation Levels

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Source of support:

This work was supported by Ministerio de Sanidad y Consumo (Instituto de Salud Carlos III, grant PI05/0783 to FCR and PI1001750 to JMU)

Background: Heart transplantation is a therapeutic option for patients with severe coronary artery disease or heart failure. One of the difficulties to overcome is the apoptosis of cardiomyocytes in the donor organ. To prevent apoptosis in the donor organ, we developed a fusion protein containing FLIP (FADD-like interleukin beta-converting enzyme (FLICE)-like inhibitory protein) to inhibit caspase-8.

Material/Methods: We linked the cDNA coding for the FLIP protein to the transduction domain of HIV (human immunodeficiency virus) to allow the protein to enter cells. The recombinant protein was used at two different concentrations, 3 nM and 30 nM, for treatment of the donor heart in rat transplantation experiments. After transplantation, apoptosis was measured by ELISA, and the levels of active caspase-3, caspase-8, Bid, and PUMA were determined by western blotting using specific antibodies.

Results: We observed that treatment of the donor organ with a solution containing this protein reduced the apoptosis level in the donor organ after 30 minutes post-transplantation as measured by the total of apoptotic cells with ELISA assay, and caspase-8 and caspase-3 activation and decreased levels of BH3-only proteins such as Bid and PUMA. Furthermore, this treatment also reduced the total tyrosine phosphorylation levels, which may be a possible measurement of lower oxidative stress levels in cardiomyocytes.

Conclusions: Protein FLIP solution reduced apoptosis at 30 minutes post-transplantation and decreased levels of several regulators of apoptosis.

MeSH Keywords: Apoptosis Regulatory Proteins • Caspase 3 • Caspase 8

Full-text PDF: <https://www.annalsoftransplantation.com/abstract/index/idArt/903913>

Background

Since the advent of cyclosporine A in the mid-1980s, heart transplantation has become a therapeutic option for patients with heart failure or severe coronary artery disease who have poor prognosis owing to severe conditions such as congestive heart failure. Ischemia and reperfusion in the donor heart triggers, in a significant proportion of cardiomyocytes, a series of effects, including apoptosis which is one of the main factors involved in transplant failure in both the short-term and long-term. Cumulative evidence indicates that impeding apoptosis confers considerable protection to donor hearts [1–5]. In fact, a correlation exists between ischemia time elapse in the donor heart and one-year survival rate in heart transplant recipients [6]. While several approaches have been used to counteract these effects [2], they all have similar limitations: they need to be rapid and they need to be able to function at low temperatures [7,8]. After surgical retrieval, donor hearts must be perfused with a cold cardioplegic solution, which can maintain the donor organ viability for several hours [9,10], after which the heart is no longer viable for transplantation. The main aim of our study was to find an approach that could slow down the aforementioned process, improve organ preservation and function, and prolong the viable period for donor hearts.

In recent years, the search for new therapeutic molecules capable of entering cells without causing significant damage has been intensified. The possibility of introducing peptides into cells using protein transduction domains (PTDs), also known as cell penetrating peptides (CPPs), has been explored. These short peptides can penetrate the plasma membrane [11–13], thereby permitting entry of proteins fused to these peptides into cells in a rapid, efficient manner [14]. Some of these sequences, such as TAT-HIV, have proven to be good options for many systems since they are rapid and function at low temperatures [15,16].

Apoptosis has been extensively studied in metazoans [17–19]; apoptosis requires energy and the damaged cells are eliminated without provoking inflammation. Apoptosis plays a role in the pathogenesis of a variety of illnesses, including cardiovascular diseases. Triggering of apoptosis occurs by intrinsic and the extrinsic pathways, both of which lead to “executioner” caspase activation and concomitant cytoskeletal and nuclear fragmentation [17–19]. The intrinsic pathway is initiated by intracellular factors such as extended DNA damage or ischemia [18–20]. The extrinsic pathway is mediated by cell surface death receptors pertaining to the tumor necrosis factor (TNF) family. In the extrinsic pathway, ligands such as FAS or TRAIL (TNF-related apoptosis-inducing ligand) interact with the death receptors FAS and DR4-5, which promotes the binding of FADD protein (Fas-associated death domain protein). FADD proteins recruit caspase-8, forming the death-inducing

signaling complex (DISC) which activates caspase-8 and, consequently, caspase-6 and caspase-7. Activation of these caspases leads to the proteolysis of substrates and to cell death [21].

One of the regulators of caspase-8 is FLIP (FADD-like interleukin beta-converting enzyme (FLICE)-like inhibitory protein) [22,23]. This protein is a specific inhibitor of caspase-8 mediated apoptosis and blocks its activation [24–26]. In humans, at least two FLIP isoforms have been described: short form (cFLIP_s, 221 aa) and long form (cFLIP_l, 480aa) [22,27]. The short form was used in this study.

Research to block apoptosis has focused mainly on the intrinsic pathway [5,28]; the role in apoptosis regulation in the process of ischemia and reperfusion has been extensively studied [19,20,29–32]. The role of the extrinsic pathway in apoptosis blockage is less well-studied. To ascertain whether blocking of the extrinsic pathway also plays a leading role in the apoptosis of cardiomyocytes from the explanted heart, we developed a fusion protein consisting of the TAT-HIV peptide linked to the cFLIP_s protein with the aim of inhibiting caspase-8 activation. The fusion protein TAT-cFLIP_s was added to the cold cardioplegic solution in which the pre-transplanted organ was being initially perfused. With this approach, the protein could act throughout the time needed for transplantation.

Our results showed that the addition of TAT-cFLIP_s to the preservation solution reduced the levels of activated caspase-3, caspase-8, and other proapoptotic regulators, such as Bid and PUMA, which suggests a prolonged of preservation time for the organ.

Material and Methods

All procedures were performed in accordance with the guidelines approved by the Spanish Ministry of Science and Technology and following the European Community Council Directive 86/609 EEC.

Animals and heart transplantation

Male Sprague Dawley rats (250–275 g) were used in this study. Heterotopic heart transplantation was performed as previously described [33,34]. Donor animals were anesthetized with isoflurane, and bilateral thoracotomy was performed. All vena cava and right atria were ligated. Ascending aorta and pulmonary arteries were cut, and the heart was extracted, washed with 5 mL of Abboplegisol (Abbot, Illinois), perfused with 2 mL of either 3 nM or 30 nM recombinant protein and stored in cold cardioplegic solution (4°C). Controls were perfused only with vehicle. Recipient animals were anesthetized [27], a long mid-abdominal incision was made and the abdominal vessels under the

renal vessels were separated. The donor pulmonary artery and ascending aorta were anastomosed to recipient inferior cava and abdominal aorta, respectively, with 6/0 Prolene sutures. Following complete irrigation, the donor heart was checked for normal beating. After 30 minutes, the donor heart was excised from the recipient, washed with 5 mL of cold Abboplegisol, cut into two pieces and immediately frozen at -80°C . Recipient rats were then euthanized. For beta-galactosidase analysis, hearts were sliced in a JungCM1800 cryostat (Leica Microsystems, Wetzlar, Germany) and stained with Sigma Beta Galactosidase Reporter Gene Staining kit from Sigma following the manufacturer's instructions. Each experiment was repeated at least three times with three animals in each condition.

Antibodies

Anti-phosphotyrosine antibody was obtained from Millipore (Billerica, MA, USA), anti-caspase-3 total, anti-cleaved caspase-3, and anti-caspase-8, as well as antibodies against Bid, Bax, and PUMA were obtained from Cell Signaling (Danvers, MA, USA).

Cell death quantification

To measure apoptosis, we used the Cell Death Detection ELISA kit from Roche (Mannheim, Germany). This system uses antibodies against histones H1, H2A, H2B, H3, and H4, and antibodies against DNA (single and double strands) permitting detection of mono- and oligo-nucleosomes and DNA. In brief, anti-histone antibodies were fixed to the plate, tissue lysates were added and incubated with antibodies against DNA coupled to peroxidase, then plates were washed; the amount of peroxidase retained with ABTS (2,2' azino-di-ethylbenzothiazoline sulfonate) as a substrate was determined by measuring absorbance at 405 nm. Results were presented as percentage of values with respect to a control.

Plasmid construction

A plasmid pRSET containing beta-galactosidase was donated by Dr. M. Morales [35]. Beta-galactosidase and the flanking cloning site were excised using Bpu 1102 and Xba I restriction enzymes. The fragment obtained was cloned into pET28a (Novagen) using the same enzymes (pET28a-WT- β -gal). To insert the TAT sequence we used two oligonucleotides, forward: TCG ATT ACG GTC GTA AAA AAC GTC GTC AGC GTC GTC GTC CGC GGC and reverse: AAT GCC AGC ATT TTT TGC AGC AGT CGC AGC AGC AGG CGC CGA GCT (Operon, Izasa, Barcelona, Spain). Oligonucleotides were denatured at 100°C for five minutes and annealed at 42°C for 60 minutes. The double-strand DNA fragment was cloned into pET28a-WT- β -gal using XhoI restriction enzyme. Resulting colonies were screened by restriction enzyme, PCR, and DNA sequencing to ensure conservation of a proper reading frame (pET28a-TAT β -gal).

To construct FLIP and TAT-cFLIP_s-containing plasmids, FLIP cDNA sequence was PCR-amplified from a rat heart cDNA library (Takara, Sigma, Japan) using Vent DNA Polymerase (New England Biolabs, Danvers, MA, USA) using the following primers: forward: 5' AAA CTC GAG ATG GCC CTG AGC ATC GTG TC 3' and reverse: 5' AAA GAA TTC TCA TGC TGA TAT TCC ACA CA 3. The PCR product was purified using the QIAEX II kit (Qiagen, Hilden, Germany), then digested with XhoI and EcoRI and purified again with the same kit following the manufacturer's instructions.

Beta-galactosidase was excised from pET28a-WT- β -gal and pET28a-TAT- β -gal, and replaced with amplified cFLIP_s cDNA. The resulting plasmids (pET28a-WT-FLIP and pET28a-TAT-cFLIP_s, respectively) were analyzed by restriction digestion and DNA sequencing using the sequencing kit Applied V3.1 (Applied Biosystems, Foster City, CA, USA) and AbiPrism 3730 sequencer.

Generation of recombinant cFLIPs

To obtain the recombinant TAT-FLIP_s protein, competent *Escherichia coli* BL21(DE3) bacteria were transformed with the recombinant plasmids pET28a-WT-cFLIP_s and pET28a-TAT-cFLIP_s. Protein expression was induced with 1 mM IPTG (isopropyl-thio-galactosidase) for four hours at 37°C with gentle shaking. Bacteria were pelleted by centrifugation at 5,000 rpm in a Sorvall 34 centrifuge, resuspended in 20 mM imidazole buffer (pH 7.0), lysed by sonication, and centrifuged again at 10,000 rpm in a SS34 rotor to clear the lysate. A Nickel-Sepharose column was then loaded in an FPLC system (LCC-500CI, Pharmacia GE Healthcare, St. Giles, United Kingdom), washed first with 20 mM imidazole buffer and then in an ascending gradient from 20 mM to 125 mM imidazole buffer. Finally, elution was performed with 250 mM imidazole buffer. Fractions were analyzed by SDS-PAGE and those containing recombinant protein were pooled and dialyzed.

Western blot analysis

To obtain soluble proteins, the samples were mixed with lysis buffer (1 g/5 mL buffer). The buffer contained 50 mM Tris pH 7.5, 1 mM EDTA, 0.1% 2-mercaptoethanol; the following protease inhibitors: 4 $\mu\text{g/mL}$ PMSF (phenylmethylsulfonyl fluoride), 5 $\mu\text{L/mL}$ aprotinin, and 5 $\mu\text{g/mL}$ leupeptin; and the following phosphatase inhibitors: 0.1M NaF, 200 μM sodium orthovanadate and 10 mM sodium pyrophosphate.

To obtain heart tissue extracts, the samples were weighed and then five volumes of lysis buffer was added. The mixture was processed in a Polytron homogenizer; the insoluble material was removed by centrifugation at 13,000 rpm for 20 minutes. One volume of 2 $\times$ Laemmli loading buffer (100 mM Tris-HCl pH 6.5, 4% SDS, 20% glycerol, 200 mM DTT, and 0.2%

bromophenol blue) was then added. The samples were boiled at 95 °C for four minutes, then analyzed by SDS-PAGE [36,37]. Samples were run in a 12% polyacrylamide gel at 150 V and then transferred to nitrocellulose membranes in 120 mM glycine, 125 mM Tris pH 8.5, and 20% methanol at 35 V overnight. Filters were saturated in TBS containing 3% BSA and incubated with antibodies at a dilution of 1/1,000 in TBS with 3% BSA. Secondary anti-rabbit antibodies or anti-mouse coupled to horseradish peroxidase diluted 1/2,000 were applied and detection was made using the Enhance Chemiluminescence kit from Amersham (GE Healthcare, St Giles, UK).

Quantitation of western blotting

For quantification, films were scanned and then subjected to band densitometry and quantification (Bio-Rad, Hercules, CA, USA). Western blots were quantified (in arbitrary units; GelPro Analyzer 3.1; Media Cybernetics); to normalize the data, we represented the percentage of arbitrary units obtained with respect to controls.

Statistical analysis

Statistical analysis was performed by commercially available software (Statgraphics Plus 5.0, StatPoint Inc., Herndon, VA, USA). Differences between groups were tested by Mann-Whitney U test. For all statistical analyses, $p < 0.05$ was considered significant.

Beta-galactosidase activity

Histochemical staining based on beta-galactosidase activity was performed on fixed tissue and stained using Sigma Beta-Galactosidase Reporter Gene Staining kit from Sigma, following the manufacturer's instructions.

Results

Apoptosis

To ascertain whether treatment with the TAT-cFLIP_s peptide would protect against apoptosis, cell death was analyzed using the Cell Death Detection ELISA test from Roche Diagnostics. This system uses antibodies against histones H1, H2A, H2B, H3, H4, and DNA detecting mono- and oligo-nucleosomes, and is used as a measurement of apoptotic cell death [38]. On analyzing the samples obtained, we observed a significant decrease in the level of apoptosis in the samples treated with the TAT-cFLIP_s peptide (Figure 1; 33% at 3 nM and 44% at 30 nM) in comparison to controls (animals treated with vehicle).

Figure 1. Total levels of apoptosis. Samples were treated with TAT-cFLIP_s peptide at 3 nM and 30 nM concentrations and apoptosis was analyzed by ELISA method. A reduction in the total level of apoptosis was observed. The data are represented as mean $\pm$ SEM of three separate experiments. Data were normalized to control (* $p < 0.01$; ** $p < 0.05$; *** $p < 0.001$).

Caspases-3 and caspase-8 activation

To ascertain which molecules were involved in the different levels of apoptosis detected, we focused on the main “executioners” of apoptosis: caspase-3 and caspase-8. Caspase-3 has clearly been shown to be responsible for apoptotic death [17] and can be regulated by caspase-8, the main target of the FLIP protein. When activated caspase-3 levels resulting from proteolysis were analyzed, the addition of TAT-cFLIP_s was found to reduce activated caspase-3 levels (Figure 2A, 2D) at both concentrations tested (3 nM and 30 nM). A reduction in activated caspase-3 levels was observed in samples treated with TAT-cFLIP_s compared to controls (35% at 3 nM and 40% at 30 nM, with respect to control animals treated with cold cardioplegic solution without fusion protein). In parallel, full-length caspase-3 levels were determined in all samples (non-processed form, Figure 2C); the differences found could be due to stochastic variation.

In addition, since TAT-cFLIPs is an inhibitor of caspase-8, inhibition of this enzyme was expected. Indeed, when activated caspase-8 levels were analyzed, levels of this molecule were found to be lower (Figure 2B, 2E). The inhibition level was 40% at 3 nM and 53% at 30 nM, concurring with the lower level of caspase-activation observed in these samples. Thus, we concluded that treatment with TAT-cFLIP_s was able to reduce caspase-8 activation levels.

Other regulators of apoptosis

One molecule involved in the connection between the extrinsic (represented by caspase-8 activation) and intrinsic pathway (through caspase-3 activation) is BH3-interacting domain

Figure 2. Western blot analysis corresponding to activated caspase-3 (A), activated caspase-8 (B), and total caspase-3 (C), with the corresponding quantification (B, D). Samples were analyzed by SDS-PAGE, transferred to nitrocellulose and blotted with antibodies against the activated form of the caspases, as well as total caspase-3 as a control. A reduction in caspase-3 and caspase-8 levels were detected. The data are represented as mean $\pm$ SEM of three separate experiments. Data were normalized to control (* $p < 0.01$; ** $p < 0.05$; *** $p < 0.001$).

death agonist (Bid), a member of the Bcl-2 family of proteins that regulates the permeabilization of the outer mitochondrial membrane. In addition, Bid is proteolyzed by caspase-8 and converted into tBid (truncated Bid) which inhibits Bcl-2 and triggers release of cytochrome C from the mitochondria [39], connecting both intrinsic and extrinsic pathways. When we measured Bid levels in the samples treated with TAT-cFLIP_s, a decrease was observed in levels of this protein (Figure 3A, 3B; 24% at 3 nM, 30% at 30 nM).

Since the decrease in apoptosis could not be completely explained by the reduction in tBid, other regulators of apoptosis were suspected to be involved. Two BH-3-only proteins were selected: Bax and PUMA. Bax is one of the main pro-apoptotic factors of the Bcl-2 family of proteins and constitutes a gateway to mitochondrial apoptosis [40]. When Bax levels in the

samples treated with TAT-cFLIP_s were analyzed, a significant decrease was observed in the samples treated with this molecule (Figure 4A, 4B). The reduction was more than 60% at both 3 nM and 30 nM compared to controls without fusion protein. Another pro-apoptotic member of the Bcl-2 protein family is PUMA, the p53 upregulated modulator of apoptosis, which is also a critical activator of apoptosis [41]. Western blotting showed a decreased amount of PUMA in the samples treated with TAT-cFLIP_s (Figure 4C, 4D), with the reduction being 65% at both concentrations tested (3 nM and 30 nM).

Phosphotyrosine levels

Levels of tyrosine phosphorylation have been linked to cell stress, transformation, and apoptosis [42]. To ascertain whether this cellular process could be involved in the action of TAT-cFLIP_s,

Figure 3. Western blot analysis corresponding to Bid protein. Samples obtained were analyzed by western blotting with antibodies corresponding to Bid. A significant reduction in the levels of Bid was observed with treatment at both 3 nM and 30 nM. The data are represented as mean $\pm$ SEM of three separate experiments. Data were normalized to control (* $p < 0.01$; ** $p < 0.05$; *** $p < 0.001$).

Figure 4. Western blot and quantification analysis corresponding to Bax (A, C) and PUMA (B, D). Samples treated with TAT-cFLIP_s at 3 nM and 30 nM or control peptide were analyzed with antibodies against the pro-apoptotic proteins Bax and PUMA. A significant reduction in the levels of these proteins was observed with the treatments. The data are represented as mean $\pm$ SEM of three separate experiments. Data were normalized to control (* $p < 0.01$; ** $p < 0.05$; *** $p < 0.001$).

we analyzed the total tyrosine phosphorylation levels with anti-phosphotyrosine antibody 4G10 after treatment with TAT-cFLIP_s and the control negative peptides. We found decreased levels of tyrosine phosphorylation in the samples treated with the TAT-cFLIP_s protein, concomitant with the decreased levels of pro-apoptotic proteins (Figure 5).

To show that the fusion protein to the TAT peptide was able to enter the donor heart, we used either TAT-beta-galactosidase

or beta-galactosidase proteins that were added to the cold cardioplegic solution at 30 nM for 30 minutes. After treatment, the tissues were fixed and stained for beta-galactosidase (Supplementary Figure 1). Only TAT-beta-galactosidase protein showed positive results indicating that the TAT sequence was able to promote the entrance of the whole protein into the heart tissues.

Figure 5. Western blot corresponding to phosphotyrosine analysis in the samples treated with 3 nM or 30 nM TAT-cFLIP_s and control peptide. A decrease in the phosphotyrosine content was observed.

Discussion

Heart transplantation is a process that has significantly improved the survival rate of patients with heart failure due to severe artery disease; however, preservation time of a donor heart is limited [20,30,43,44], lasting only a few hours, and one of the main problems to be addressed is apoptosis suffered by cardiomyocytes in the donor organ [2,7,9]. With this in mind, and in an attempt to improve the heart transplantation process, we aimed to ascertain whether anti-apoptotic protein cFLIP_s could in fact lower apoptosis levels. cFLIP_s has been linked to apoptosis regulation through the extrinsic pathway [22,24,26,45–47], and consequently, also associated with caspase-3 inhibition; however, this protein cannot reach the cytosol of cells unless specific sequences, such as signal transduction domains, are fused to the sequence of cFLIP_s [12,13]. This method has been used effectively to deliver proteins to the cytosol [13] and, in our case, proved to successfully transport cFLIP_s to cells. Consequently, we prepared recombinant protein cFLIP_s fused to the cell-penetrating peptide from HIV-TAT [12]. This molecular tool was used to ascertain whether apoptosis was indeed inhibited. Apoptosis was measured using different methods, including Roche Cell Detection kit and activation of pro-apoptotic caspases such as caspase-3 and caspase-8. The results showed that under these conditions a significant decrease in apoptosis activation occurred. We also found a decrease in activation of caspase-3 and caspase-8 in the transplanted hearts. Previous studies have shown that cFLIP_s can be an effective target of anti-cancer therapy [22,45]. We confirmed that the TAT sequence was effective in promoting protein penetration in cells as was evident from beta-galactosidase staining (Supplementary Figure 1). Our data showed, for the first time, that this recombinant protein could improve the preservation of the donor organ (which currently does not exceed

a few hours) and is consistent with the idea of cFLIP_s being a putative target for also modulating apoptosis in cardiac tissue.

The mechanism through which cFLIP_s exerts its effect on cardiac tissue was also analyzed in our study. Of particular significance was the Bid molecule, which connects extrinsic and intrinsic pathways [48]; our data showed a consistent decrease in Bid levels detected in the samples treated with TAT-cFLIP_s, which indicated that the effect of the observed apoptosis inhibition occurred mainly through Bid.

Other molecules involved in apoptosis regulation, such as Bax and PUMA, were also analyzed. Albeit with some differences, a significant decrease in the apoptotic regulators tested in both proteins was observed in all cases analyzed, in accordance with the inhibition in caspase-3 and caspase-8.

The role of phosphotyrosine content in the treatment with TAT-cFLIP_s in the donor organ was also assessed. Extensive research has documented an increase in phosphotyrosine content as a consequence of not only mitogen stimulation and differentiation, but also oxidative stress [49], being a partial measurement of potential tissue damage that could activate cell death [17]. In our study, a decrease in phosphotyrosine content was observed in the hearts treated with TAT-cFLIP_s, which was consistent with the aforementioned reports. This suggests that the mechanism of protection will include, at least in part, activation of tyrosine phosphatase and/or inhibition of tyrosine kinases, although this has yet to be determined.

According to our data, TAT-cFLIP_s appears to be a good tool to decrease apoptosis in donor organs. Inhibition would seem to occur through the regulation of the extrinsic pathway, although the exact molecular regulation requires further investigation. Consequently, the TAT-cFLIP_s approach to improving organ transplantation appears to be a promising therapeutic option; however, more studies are required to confirm this possibility.

Conclusions

TAT-FLIPs was able to inhibit apoptosis in rat hearts at 30 minutes post-transplantation, as well as decrease caspase-3 and caspase-8 activity. It also inhibited apoptosis regulators such as Bax and PUMA.

Acknowledgments

We thank Professor Joan Gil for helpful discussion and Miss Christine O'Hara for language revision. We also thank Maria Eugenia Ureña for technical and editorial help. We dedicate this article to our colleague and friend José Carreras who passed away when this manuscript was in preparation.

Supplementary Figure

Supplementary Figure 1. Histochemical staining based on beta-galactosidase activity. Animals were treated with cold cardioplegic solution containing 30 nM of TAT-beta-galactosidase or beta-galactosidase alone for 30 minutes. After treatment, the tissue was fixated and stained using the Sigma Beta-Galactosidase Reporter Gene Staining kit from Sigma following the manufacturer's instructions. (A–C) Slices of rat heart treated with TAT-beta-galactosidase; (D–F) slices of rat heart treated with beta-galactosidase. Magnification: A, D, 50 $\times$; B, E, 100 $\times$; C, F, 400 $\times$.

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