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Anti-Apoptotic Effects of Cellular Therapy: VTL C3A Cell-Secreted Factors Reduce in vitro Hepatocellular Injury via Multiple Mechanisms

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VITAL THERAPIES® TARGETING LIVER DISEASE

BACKGROUND

Hallmarks of alcoholic hepatitis (AH) are increased hepatocellular death, increased liver dysfunction and further inflammatory responses if dying cells are ineffectively cleared.[1,2] Vital Therapies, Inc. (VTL) is clinically evaluating an investigational liver treatment (ELAD System) using VTL C3A cells in the treatment of severe AH (sAH). We previously reported that conditioned medium (CM) from VTL C3A cells grown in a three-dimensional bioreactor contains hepatocyte mitogens (amphiregulin, TGF-α, HGF, HB-EGF, and PDGF-BB) and can inhibit Fas-induced apoptosis in primary human hepatocyte (PHH) cultures, as measured by caspase 3/7 activity and annexin V staining;[3] however, the mechanism was previously unknown.

OBJECTIVES

The purpose of this study was to determine the mechanisms by which VTL C3A cell-secreted factors in CM can promote hepatocyte survival in a model of Fas-induced apoptosis.

We propose three independent pathways by which CM may offer hepatoprotective effects:

(i) epidermal growth factor receptor (EGFR) activation; (ii) soluble Fas (sFas) competition for Fas ligand (FasL); and (iii) reducing oxidative stress.

MATERIALS & METHODS

Vital Therapies' (VTL) ELAD® System is an investigational extracorporeal human hepatic cell-based liver treatment comprised of four metabolically-active ELAD cartridges with ancillary device components and support circuitry intended to continuously treat subjects with liver failure secondary to acute hepatocellular insult and alcohol use for up to 5 days**.**

A primary human hepatocyte (PHH) apoptosis model was adapted from Berasain.[4] Apoptosis was induced in PHH (Gibco) using anti-CD95 (Fas) antibody (EOS9.1, eBioscience) following a 3-h incubation with Williams E medium (w/supplements, w/o dexamethasone [Gibco]) or ELAD CM prepared by static incubation of Williams E medium in a mature ELAD C3A cell cartridge. Recombinant human soluble Fas (sFas) and amphiregulin (R&D Systems), and EGFR-inhibitor (EGFR-I) canertinib (abcam) were also used as treatments to establish dependence of the model.

Caspase-Glo 3/7 Assay (Promega) and annexin V (Roche) were used to measure apoptosis.

Western Immunoblotting. All antibodies were from Cell Signaling.

Metabolomic Profiling. Spent media from ELAD cartridges, plasma, and/or ultrafiltrate from subjects surviving ≥91 d of the VTI-208 clinical study (n=8 ea. Controls & ELAD-treated) were retrospectively analyzed by global, unbiased metabolomics profiling and reported as relative-fold changes against various comparators for statistical significance (p≤0.05).

Reduced glutathione (GSH) to oxidized glutathione (GSSG) ratios were determined using a chemiluminescent GSH Glo assay (Promega).

RESULTS

Caspase Activity. Addition of ELAD CM significantly inhibited apoptosis as measured by caspase-3/7 activity (Fig.1) and annexin V staining (Fig.2), confirming previously reported results.

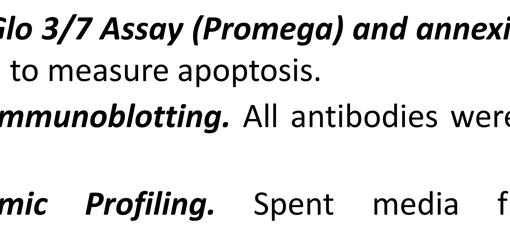


Fig 1. ELAD CM Reduced Caspase Activity. Apoptosis, as measured by caspase activity, was reduced in both untreated and Fas agonist-treated PHH cultures in the presence of ELAD CM. Error is SD of n=8 wells in 96-well format. (*** p<0.001 for ELAD-treated vs. untreated or Fas agonist-treated. One-way ANOVA with Tukey post-hoc test).

RESULTS (cont.)

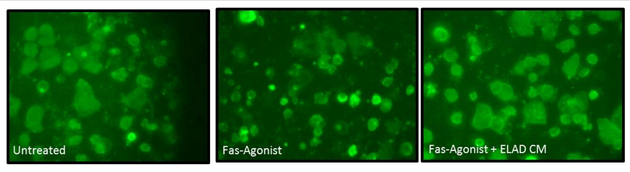


Fig 2. ELAD CM Reduced Apoptotic Phenotypic Shift in PHH. Cobblestone morphology and minimal fluorescence of untreated controls (left). Morphology was maintained in ELAD CM-treated PHH cell cultures (right) relative to Fas agonist-treated cells (center). Agonist-treated cells also show greater annexin V fluorescent staining.

Cleavage Products, Western Immunoblot. Caspase-8 and poly (ADP-ribose) polymerase (PARP) cleavage products , indicative of apoptosis, showed patterns consistent with those of the Caspase-Glo assay and annexin V staining. Fas agonist-treated PHH lysates showed increased caspase 8 and PARP cleavage products, whereas lysates from PHH treated with Fas agonist in the presence of CM showed a reduction of caspase 8 and PARP cleavage compared to controls. Further, addition of the EGFR-I canertinib, to Fas agonist/CM-treated PHH produced cleavage product levels similar to Fas agonist alone, suggesting EGFR activation as a mechanism for the reduced apoptosis.

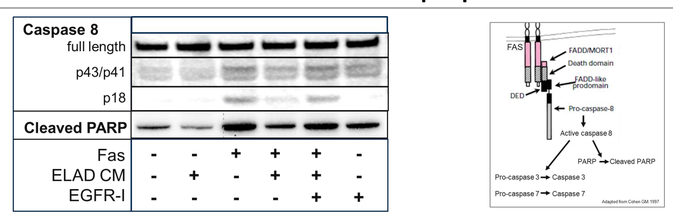


Fig 3. ELAD CM Reduced Caspase 8 and PARP Cleavage. Fas agonist-treated PHH lysates showed both increased caspase 8 (p-18) and PARP cleavage products, whereas lysates from PHH treated with Fas agonist in the presence of CM showed a reduction of both cleavage products compared to controls. EGFR inhibition reversed the protective effect.

EGFR Signaling, Western Immunoblot. Phosphorylation of proteins known to be associated with EGFR activation (e.g. $EGFR_{TYR\ 1068}$, MEK 1/2, ERK 1/2, and STAT3)[6] were increased in lysates of CM-treated PHH and were decreased in samples treated with the EGFR-I canertinib (Fig.4).

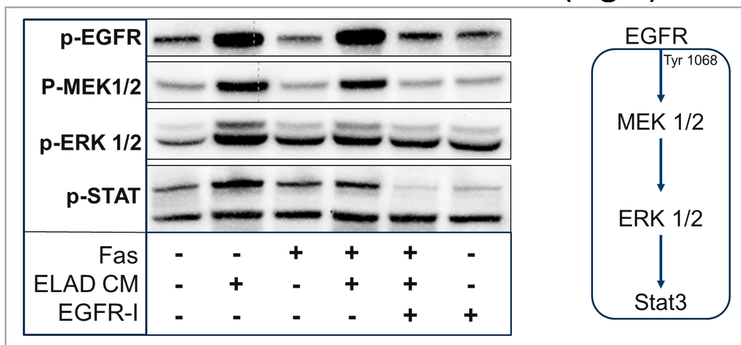


Fig 4. ELAD CM Induced Phosphorylation of Molecules Associated with EGFR Signaling. EGFR, MEK1/2, ERK1/2 and STAT3 were phosphorylated in lysates from ELAD CM-treated PHH. EGFR-inhibitor canertinib reduced this signal, however, not completely.

Amphiregulin Reduces Caspase Activity. Treatment with recombinant human amphiregulin reduced PHH apoptosis, measured by caspase activity, an effect blocked when canertinib was added to the treatment (Fig. 5). This hepatoprotective effect was less than that of CM, suggesting that an additional mechanism and/or EGFR ligand may be involved.

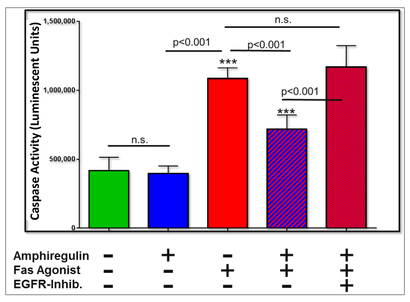


Fig 5. Amphiregulin Reduced Caspase Activity. Fas agonist-treated PHH lysates showed reduced apoptosis as measured by caspase activity in the presence of amphiregulin. Addition of the EGFR-inhibitor canertinib blocked the effects of amphiregulin. (ANOVA, Tukey's post-hoc test)

sFas Secretion by VTL C3A Cells. VTL C3A cells produce sFas in monolayer and in ELAD cartridges (unpublished). Recombinant human sFas was effective in reducing apoptosis in PHH (Fig.6), supporting secretion of sFas by VTL C3A cells as an additional and novel factor contributing to survival of PHH in this Fas-induced apoptosis model.

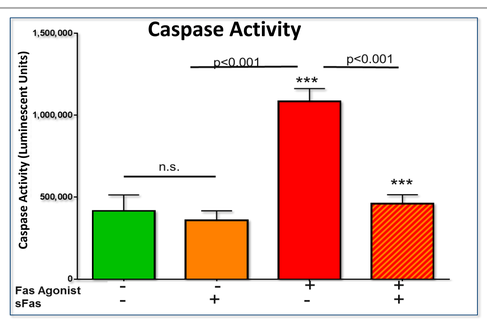
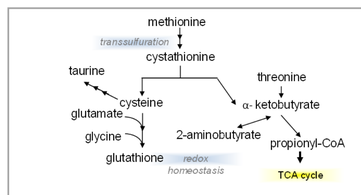


Fig 6. sFas Reduced Caspase Activity. Fas agonist-treated PHH lysates showed reduced apoptosis as measured by caspase activity in the presence of sFas. (ANOVA, Tukey's post-hoc test)

RESULTS (cont.)

Metabolomic Profiling. Of the 241 targets identified at clinical baseline, 38 increased and 203 decreased in plasma from ELAD-treated subjects vs. Controls. The pattern of downregulation of tricarboxylic acid (TCA) cycle intermediates in plasma from ELAD-treated subjects vs. Controls is suggestive of the VTL C3A cells producing a more reducing (anti-oxidant) environment (Fig. 7).



- Decreases levels of methionine intermediates and increased levels of cystathione suggest increased sulfuration of methionine
- Decreased levels of taurine suggest increased hepatic glutathione synthesis

	Treated						Untreated					
Biochemical Name	T-D3 T-D0	T-D5 T-D0	T-D7 T-D0	T-D14 T-D0	T-D28 T-D0	T-D91 T-D0	UT-D3 UT-D0	UT-D5 UT-D0	UT-D7 UT-D0	UT-D14 UT-D0	UT-D28 UT-D0	UT-D91 UT-D0
methionine	1.03	0.89	0.92	0.89	0.84	0.58	1.21	1.28	0.98	0.71	0.39	0.38
N-acetylmethionine	0.12	0.14	0.18	0.82	0.97	1.49	1.33	1.3	1.31	1.07	0.73	1.05
N-formylmethionine	0.73	0.74	0.75	0.9	0.93	0.85	1.1	1.16	1.11	1.01	0.82	0.83
S-methylmethionine	0.49	0.19	0.14	0.37	0.61	0.26	1.13	0.52	0.48	0.72	0.54	0.91
methionine sulfone	1.14	1.14	1.2	1.41	1.53	1.7	1.12	1.22	1.45	1.67	1.91	1.62
cystathionine	4.88	4.83	5.87	1.07	0.58	0.16	1.14	1.25	1.19	0.84	0.39	0.24
alpha-ketobutyrate	1.38	1.9	1.63	1.24	0.74	0.54	0.83	1.47	1.31	1.09	1.24	1.29
taurine	0.7	0.71	0.61	0.64	0.9	1.18	0.89	0.97	0.87	0.9	1.16	1.03

Fig 7. A Pattern of TCA Cycle Intermediate Downregulation in ELAD-Treated Subjects. Dark green shading highlights the greater degree of TCA cycle intermediates significantly down regulated in ELAD-treated subjects. This is consistent with increased glutathione synthesis. Glutathione, an important cellular antioxidant, is not directly measurable in plasma.

GSH/GSSG Changes in Fas-Induced Apoptosis of PHH. Consistent with the data above, PHH exposed to CM were shown to have over 5-fold increased molar ratios of reduced glutathione (GSH) to oxidized glutathione (GSSG) (Fig. 8).

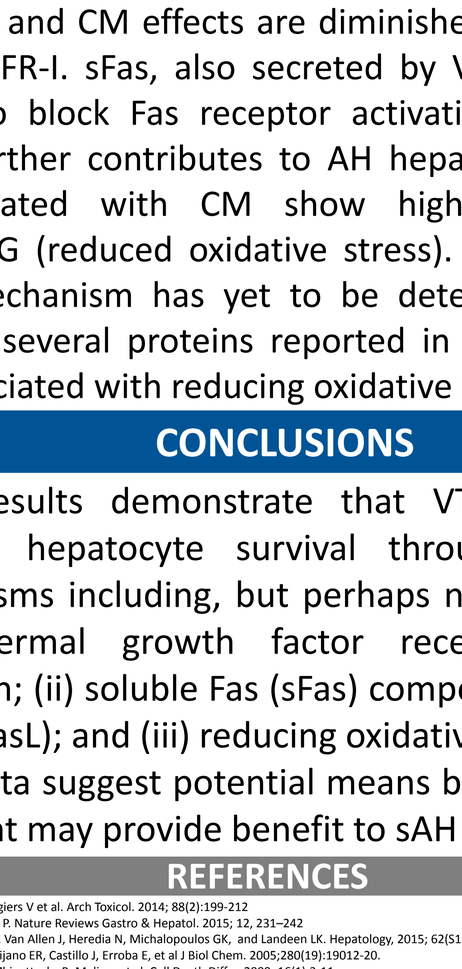


Fig 8. ELAD CM Increased the Ratio of Reduced Glutathione to Oxidized Glutathione in Cultured PHH. ELAD CM treatment increased the amount of reduced glutathione relative to oxidized glutathione over 5-fold above untreated cells, in both Fas-induced cells and non-induced (untreated) cells.

DISCUSSION

Excessive hepatocellular death and resulting increased liver dysfunction and exacerbated inflammation are key aspects of AH. Preventing hepatocyte death presents an important target for therapeutic intervention. These studies show that factors secreted from metabolically-active VTL C3A cells promote survival of PHH, in a Fas-induced apoptosis model, by impacting multiple pathways; something that non-cell-based therapies are unlikely to achieve. EGFR activation, specifically by the ligand amphiregulin, is key to hepatocyte survival and liver regeneration.[7] ELAD CM contains amphiregulin,[3] activates known signaling molecules in the EGFR pathway, and CM effects are diminished by addition of an EGFR-I. sFas, also secreted by VTL C3A cells, serves to block Fas receptor activation. Oxidative stress further contributes to AH hepatocyte death. PHH treated with CM show higher ratios of GSH/GSSG (reduced oxidative stress). Although the exact mechanism has yet to be determined, CM contains several proteins reported in the literature and associated with reducing oxidative stress.

CONCLUSIONS

These results demonstrate that VTL C3A cells promote hepatocyte survival through multiple mechanisms including, but perhaps not limited to, (i) epidermal growth factor receptor (EGFR) activation; (ii) soluble Fas (sFas) competition for Fas ligand (FasL); and (iii) reducing oxidative stress.

These data suggest potential means by which ELAD treatment may provide benefit to sAH subjects.

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