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ELAD C3A CELLS MAY IMPACT LIVER REGENERATION THROUGH SECRETED FACTORS

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BACKGROUND

The normal liver possesses enormous capacity to regenerate and replace tissue loss when damaged. However, hepatocytes of patients with hepatitis due to chronic alcohol consumption, viral infection, or fulminant toxicants, have both diminished replicative capacity and increased apoptotic cell death[1].

Liver regeneration is a highly orchestrated event involving multiple pathways and cell types. Regeneration is initiated by an innate immune response, followed by hepatocyte survival, proliferation and maturation, then matrix remodeling and cessation of proliferation[2,3,4].

VTL C3A cells have been shown to respond to pro-inflammatory cytokines and key mediators of the acute-phase response[5], found elevated in alcohol-induced liver decompensated (AILD) patients, by expression of anti-inflammatory proteins. This has the potential to contribute to the first stage of regeneration.

Our present studies demonstrate a potential role for VTL C3A cells' factors in a subsequent stage of liver regeneration, that of promoting cell survival and proliferative capacity of various liver cell types.

OBJECTIVES

The purpose of these studies was to evaluate the ability of VTL C3A cells to secrete factors reported in the literature as having a beneficial effect on hepatocyte survival, replication and/or liver regeneration. Then, finding such factors, to evaluate the effects of selected factors on various liver cell types.

MATERIALS & METHODS

Vital Therapies' (VTL) ELAD® System is an investigational 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.

ELAD C3A cell cartridge spent media were assayed using contracted ELISA multiplex (Myriad) or chemiluminescent multiplex array detection (Aushon Ciraplex) assays for known mitogenic, angiogenic and other regenerative factors.

A primary human hepatocyte (PHH) apoptosis model was adapted from Berasain[6]. 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 conditioned media (ELAD CM) prepared by static incubation of Williams E medium in a mature ELAD C3A cell cartridge. Apoptosis was measured by Caspase-Glo 3/7 Assay (Promega), annexin V (Roche) and Western immunoblot (primary antibodies, Cell Signaling).

A human aortic endothelial cell (HAEC) angiogenic factor model was developed as a surrogate for liver sinusoidal EC (LSEC) by co-culture in Transwells with VTL C3A cells or treated with ELAD CM prepared by static incubation of EGM-2 media (Lonza) in a mature ELAD cartridge. Cumulative expression of selected angiogenic factors was measured in supernatants at 24, 48 and 72 h by Aushon Ciraplex.

RESULTS

ELAD C3A cell cartridge spent media

Evaluation of ELAD spent media (media collected from mature ELAD cartridges, maintained under flow, at steady-state conditions) showed that the VTL C3A cells produce a number of recognized growth and angiogenic factors.

The measured concentrations were compared to concentrations reported in the literature of normal healthy individuals (**Table 1**).

Table 1. Growth and Angiogenic Factors in ELAD Spent Media

Factor	Normal Serum Conc.	ELAD Spent Medium Conc.[1]
Hepatocyte Growth Factor (HGF)	574 pg/mL	50 pg/mL
Transforming Growth Factor-α (TGFα)	150 pg/mL	400 pg/mL
Amphiregulin (AR)	200 pg/mL	300 pg/mL
Heparin-binding EGF-like Growth Factor (HB-EGF)	5 pg/mL	5.5 pg/mL
Platelet-derived Growth Factor-BB (PDGF-BB)	8,500 pg/mL	190 pg/mL
Vascular Endothelial Growth Factor (VEGF)	150 pg/mL	117,000 pg/mL
Vascular Endothelial Growth Factor-C (VEGF-C)	2,800 pg/mL	140 pg/mL
Placental Growth Factor (PlGF)	8 pg/mL	850 pg/mL
Angiopoietin-2 (ANG2)	1,100,000 ng/mL	0.8 ng/mL
Stem Cell Factor (SCF)	3,300 pg/mL	75 pg/mL
Erythropoietin (EPO)	4-27 mU/mL	200 mIU/mL

[1]*Experimentally determined steady-state ELAD growth system concentrations (at 5 mL/min single-pass perfusion flow rate)*

To assess the potential effects of these factors on the various cells of the liver, a series of cell-based models were developed.

PHH apoptosis model. ELAD CM administered 3 h prior to challenge of PHH cultures with a Fas-agonist antibody significantly reduced Fas-mediated apoptosis, as measured by caspase activity (**Fig. 1**). ELAD CM also reduced spontaneous apoptosis in untreated hepatocytes.

ELAD CM-treated PHH maintained a more normal size and cobblestone morphology vs. Fas-agonist-treated PHH, as visualized by annexin V staining (**Fig. 2**).

Western immunoblots showed phosphorylation of signaling proteins associated with the EGFR (AKT, ERK1/2 and STAT3) in lysates from cells treated with ELAD CM or ELAD CM plus Fas-agonist. However, there was also phosphorylation of these same signaling proteins in the untreated control and to a varying extent the Fas-treated cells (**Fig. 3**).

HAEC angiogenic factor model. ELAD CM administered daily over 72 h significantly increased PlGF secretion by HAEC in a time-dependent manner (**Fig. 4**).

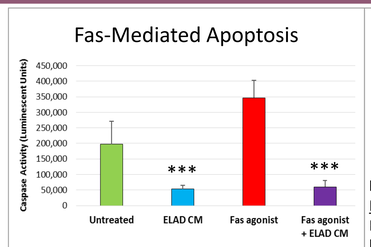

Fig 1. ELAD CM Reduces Apoptosis in PHH. Caspase activity was reduced in both untreated and Fas agonist-treated PHH cultures in the presence of ELAD CM. Error is SD of n=8 wells in 96-well format. (*** p<0.001 for all comparisons except two ELAD- treated vs each other. One-way ANOVA with Tukey post-hoc test)

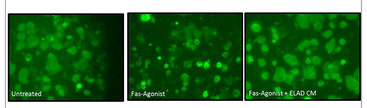

Fig 2. ELAD CM Reduces Apoptosis Phenotypic Shift. Cobblestone morphology and minimal fluorescence of untreated controls (left). Morphology was maintained in ELAD CM-treated PHH cell cultures (right) relative to Fas agonist-treated cells (center) agonist-treated cells also show greater annexin V fluorescent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

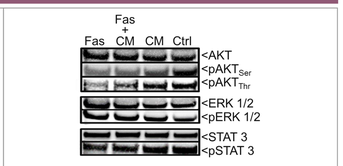

Fig 3. ELAD CM Induces Phosphorylation of Molecules Associated with EGFR Signaling. AKT, ERK1/2 and STAT 3 were phosphorylated in lysates from ELAD CM-treated PHH. However, signal from media background prohibits definitive conclusions.

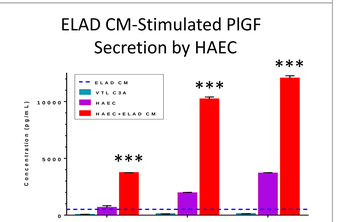

Fig 4. ELAD CM Increases PlGF Secretion by HAEC. HAEC cultures secrete significantly more PlGF in the presence of ELAD CM than EGM-2 media. Error bars are SD of n=2 replicates in 24-well format. (*** p<0.001 vs. HAEC. Two-way ANOVA, repeated measure)

DISCUSSION

Liver regeneration is a highly orchestrated event involving multiple pathways and cell types. Metabolically-active VTL C3A cells offer the potential of contributing to liver regeneration by impacting these multiple cell types and pathways in ways that non-cell-based therapies are unlikely to achieve.

These studies highlight eleven factors secreted by VTL C3A cells with recognized roles in cell growth, survival, regeneration, and hematopoiesis. The steady-state amount of each factor produced by four ELAD cartridges during manufacturing is compared in **Table 1** with normal serum values. Pharmacokinetic modeling of expected plasma concentrations in ELAD-treated subjects is not offered here. In future studies, plasma samples from the VTI-208 clinical trial will be evaluated to determine actual concentrations of selected factors achieved during treatment.

To begin to assess the potential effects of these factors on cells of the liver and to better understand the mechanisms of action of the ELAD System, ELAD CM was administered to PHH in culture. ELAD CM was found to promote survival in both untreated cells and those induced toward apoptosis by a Fas-agonist antibody (**Fig. 1**).

A similar model was shown to be dependent upon AR[6], the most necessary EGFR-ligand for liver regeneration after partial hepatectomy[7]. However, AR was not protective at 20 nM in our model (data not shown [DNS]). To determine if the pro-survival effect of ELAD CM was mediated by EGFR activation, we evaluated cell lysates from untreated and Fas-agonist-treated VTL C3A cells with and without ELAD CM, by Western immunoblot (**Fig. 3**). We found phosphorylation of AKT, ERK1/2, and STAT3, suggesting activation of the EGFR. The VTL C3A cells secrete a number of EGFR ligands (TGFα, AR, HB-EGF). However, high background in the media control, likely from insulin and other growth factors, prevent more definitive conclusions from the signaling pathway data about the exact mechanism(s) by which ELAD CM is protective towards PHH.

The pro-PHH-survival effect of ELAD CM is consistent with data showing CM from HepG2 cells (parental cell line to C3A cells) contains essential factors to support human fetal hepatocyte growth in culture[8].

LSEC and bone marrow progenitor cells of LSEC (BMSPC) have been shown to participate in liver regeneration by increased production of HGF in response to hepatic VEGF[9]. The effects of ELAD C3A cell-secreted VEGF was evaluated in an HAEC co-culture surrogate model of LSEC (due to greater availability of HAEC). Although HGF was not significantly increased (DNS), secretion of PlGF increased 5-fold over untreated HAEC, 24 h after administration of ELAD CM. The HAEC continued to produce increased PlGF in the presence of ELAD CM for the 72-h length of the model (**Fig. 4**). PlGF is purported to recruit VEGFR1$^+$ stem cells from bone marrow for organogenesis[10].

Both SCF and EPO work synergistically with G-CSF; SCF to induce proliferation in cholangiocytes and hepatocytes, and EPO to increase survival in patients with decompensated cirrhosis[11]. G-CSF secretion increases in VTL C3A cells in response to IL-1β and IL-6 (DNS).

CONCLUSIONS

VTL C3A cells produce a variety of secreted factors with well-established roles in cell growth, survival, regeneration, and hematopoiesis. Our cell-based models prevented PHH apoptosis and enhanced HAEC PlGF secretion. This may facilitate liver regeneration, directly by stimulation of hepatocytes, or indirectly by interactions with other resident cell populations during ELAD treatment.

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