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Effect of Purified Fractions from Cell Culture Supernate of High Density pre-B Acute Lymphoblastic Leukemia Cells (ALL3) on the Growth of ALL3 Cells at Low Density

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Abstract

The mechanisms underlying the aberrant growth and interactions between cells are not understood very well. The pre-B acute lymphoblastic leukemia cells directly obtained from an adult patient grow very poorly or don't grow at all at low density (LD), but grow better at high starting cell density (HD). We found that the LD ALL3 cells can be stimulated to grow in the presence of diffusible, soluble factors secreted by ALL3 cells themselves growing at high starting cell density. We then developed a biochemical purification procedure that allowed us to purify the factor(s) with stimulatory activity and analyzed them by nanoliquid chromatography-tandem mass spectrometry (nanoLC-MS/MS). Using nanoLC-MS/MS we have identified several proteins which were further processed using various bioinformatics tools. This resulted in 8 protein candidates which might be responsible for the growth activity on non-growing LD ALL3 cells and their involvement in the stimulatory activity are discussed.

Keywords

Acute lymphoblastic leukemia; Collective Behavior; Protein purification; Secretome; Serum Removal; Quorum Sensing

Introduction

Collective behavior is an essential feature involved in regulating many biological processes such as cell migration, stem-cell maintenance, maintenance of proper organ size, immune system regulations, hematopoiesis homeostasis and regeneration in higher organisms [1-9]. These beneficial collective behaviors of single cells are coordinated using 'autocrine' and/or 'paracrine' factors [10]. Bacteria utilize these quorum-sensing secretory molecules to 'count' their numbers to perform complex behaviors similar to higher organisms. Population

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level collective behaviors in bacteria include formation of complex biofilm, antibiotic production, motility, sporulation, virulence, competence, conjugation and symbiosis [11-14].

Cancer cells use collective behavior to function as a closely integrated population to maintain abnormal cell proliferation, resistance to apoptosis, invasion, metastasis, manipulating immune responses, developing therapeutic resistance, and extensive metabolic reprogramming [8, 9, 15, 16]. The underlying biochemical and biological quorum-sensing mechanisms in cancer cell populations are still poorly understood [8, 17-19]. Bacteria use small molecules or peptides to coordinate their collective quorum sensing behaviors and act as multicellular organisms. How the cancer cells regulates their number and the quorum sensing molecules involved in the regulatory mechanisms are not well understood. There are several mechanisms of cell-cell communications. A direct contact between cells by integral membrane proteins is one of them. Another mechanism is indirect contact via the extracellular matrix. Cells also communicate with each other via soluble diffusible factors or via membrane vesicles.

Here we have been studying a new Ph+ ALL cell line (ALL3) recently established from the leukemic cells growing as an ascitic cells in the pleural fluid of an adult patient with Ph+ ALL who died of his disease shortly after the cells were obtained. The ALL3 cell line used in this study provided a unique opportunity to investigate the mechanisms regulating the growth of these malignant cells that closely simulate the pleural fluid ecosystem in which they were growing in the patient. In contrast to many other long-established human or murine BCR-ABL driven leukemic cell lines, ALL3 cells do not form colonies in methylcellulose, do not grow in liquid culture at low cell densities (~5000 - 10,000 cells/ml) (LD), and grow increasingly faster at progressively higher cell densities (HD) between 20,000 cells/ml and $3 - 4 \times 10^5$ cells/ml without stimulation by any growth factors (GFs) [8]. The ALL3 cells are unresponsive to any known hematopoietic cytokines, produce no clones in semi-solid media, not even tiny ones, and don't grow as single cells in 60-well single cell cloning plates. The ALL3 cells shortly enter apoptosis and die at LD, but the apoptosis of LD ALL3 cells can be repressed in the presence of the HDSN. Labeling studies with Ki67, BrdU or EdU showed that the LD ALL3 cells are poised to begin proliferating but cannot do so without being triggered by HDSN from ALL3 cells or some other normal or leukemic cells growing at HD [8]. The cell-free supernates containing diffusible, soluble and secretory factors from ALL3 cells grown at high starting cell densities (HDSN) were found to stimulate the growth of the ALL3 cells at LD at which they otherwise don't grow. The importance of the "membrane vesicles" or "exosomes" and their stimulatory activity on the non-growing LD ALL3 cells are reported elsewhere [9].

This study focused on the effects of soluble factors and their stimulatory activity on the growth of non-growing LD ALL3 cells. Soluble secretory proteins are important mediators of intercellular communications. Nerve growth factor was amongst the first one discovered by Rita Levi-Montalcini and Stanley Cohen [20-23]. The stimulatory diffusible and soluble factors responsible for the growth of the non-growing LD ALL3 cells are present in the secretome of the ALL3 cells growing at high starting cell densities. To identify the unknown stimulatory factor(s) we grew the HD ALL3 cells in different liquid culture media that contained various concentrations of FBS. We found that lower percentage (~5% FBS) of

serum used to grow the HD ALL3 cells were used with trade off in terms of lower stimulatory activity on the LD ALL3 cells compare to 10% FBS grown high density ALL3 cells. We then developed a purification procedure that allowed us to remove the most abundant FBS proteins. The highly purified stimulatory factor(s) was/were then analyzed by nanoLC-MS/MS. The proteins identified and possibly responsible for the stimulatory activity are discussed.

Methods

ALL3 Cells

The human p190^{BCR-ABL} driven ALL cells line (ALL3) was derived from the rapidly growing Ph+ ALL leukemic cells in ascitic form in the pleural fluid of a patient with widely disseminated Ph+ ALL who died shortly thereafter. Multiple aliquots of ALL3 cells were frozen to preserve the cells' condition as closely as possible to their status in the pleural fluid. When experiments were planned, an aliquot was thawed about a month or so ahead of time as it took a few weeks or longer for the majority of cells surviving the freeze/thaw procedures to resume growing at about their original rate in the pleural fluid and immediately after collection of the thoracentesis fluids *in vitro* [8]. A portion of the thawed cells was refrozen for future use. During the course of a series of experiments the cells were passaged serially for about 4-6 months during which they usually maintained their original growth characteristics, but with longer passage they sometimes began to adapt to the liquid culture conditions and started to grow at lower cell densities at which they would not grow originally. During this optimal experimental period the cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah, USA), 1% penicillin-streptomycin solution (PSN; 10,000 I.U. penicillin and 10,000 µg/ml streptomycin) (Corning # 30-001-CI, Fisher Scientific, Pittsburgh, PA, USA), 1% sodium pyruvate (Mediatech Inc, Manassas, VA, USA # 25-000-CI), 1% HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) (Mediatech # 25-060-CI), 1% non-essential amino acids (NEAA; Mediatech #25-025) and 0.1% β-mercaptoethanol (BME) (Life Technologies, Grand Island, NY, USA # 21985-023). Hereafter, we will use the term ALL3 media to describe the above culture medium used to culture the ALL3 cells. The Cellgro was purchased from Mediatech and QBSF-60 from Quality Biological.

Collection of Supernatant from ALL3 Cells

For protein purification, the HD ALL3 cells were cultured with 3 to 5% FBS in ALL3 media and grown for 48–72 h at 37°C in a 5% CO₂ atmosphere for collecting supernatant. The cell cultures were harvested after 3-4 days of growth and the cells were centrifuged at 2,000× *g* for 10 min. The supernatant was collected and filtered using Millipore 0.22 µm 50-ml filter tubes. The filtered-cell free supernatant was used either for biochemical purification or assays.

[³H]-Thymidine Incorporation Assay

The supernates from cells growing at high densities were collected as described above. For growth assays, cells were counted using the trypan-blue dye exclusion method, and cells

equivalent to 5,000- 10,000 cells/ml (LD ALL3 cells) were placed into 15-ml falcon tubes. The adjusted ALL3 cells at the LD were washed with fresh ALL3 media once, and replaced with ALL3 HDSN or protein fractions collected after biochemical purification steps or with plain ALL3 media (as negative control). In each experiment the cell suspension volume was 6-7 ml. The fractions were suspended in 6-7 ml of ALL3 media and syringe filtered before suspending the LD ALL3 cells. Then, the LD cells were resuspended thoroughly and dispensed into two 6-well flat bottom plates with 3 ml cell suspension per well in each plate and kept at 37°C, 5% CO₂ in an incubator until harvesting them for assay. The day before harvesting the cells on a filter plate, 200 µl of cell suspensions were taken from the 6-well plate and seeded in triplicate in 96-well round bottom plates. Then, these cells were incubated with 20 µl (for each well) of 0.3 µCi of [³H]-thymidine (PerkinElmer Life Sciences, Shelton, CT, USA), and the cells were incubated for 18 h at 37°C and 5% CO₂. Next day, cells were harvested on Unifilter GF/C 96-well plates (Perkin Elmer Life Sciences # 1450-521) using Unifilter-96 cell harvester (Perkin Elmer Life Sciences # 961961) as described by manufacturer. The plates were allowed to air dry, and 20 µl Microscint-20 fluid (Perkin Elmer Life Sciences # 6013621) was added, the plate was covered with transparent top-seal and opaque back-seal, and [³H]-thymidine radioactivity was measured in Top Count Microplate Scintillation Counter (Perkin Elmer Sciences). This assay system was used to determine stimulatory activity of HDSN and other biochemical purified fractions on the growth of the LD ALL3 cells.

Biochemical Purification

Buffers

Buffer A consists of 25 mM Tris-HCl, 35 mM KCl, 5% Glycerol, pH8.0. Buffer B consists of 25 mM Tris-HCl, 1M KCl, 5% Glycerol, pH 8.0. Buffer C consists of 25 mM Tris-HCl, 35 mM KCl, 5% Glycerol, pH 9.2. Buffer D consists of 25 mM Tris-HCl, 200 mM NaCl, 15% Glycerol, pH 8.0.

Preparatory Procedure for HiTrap-Q Chromatography

The AKTA FPLC apparatus (GE) system was used for all the biochemical purification steps. The filtered HDSN containing 5% FBS from ALL3 cells grown at high starting cell densities was directly applied to a HiTrap-Q column (GE # 17-1154-01; 5ml) previously equilibrated with buffer A. The column was washed with five column volume with buffer A, and then bound material was eluted with 50 ml (10 column volume) gradient of a 0.025 - 1 M KCl gradient at a flow rate of 150 ml/h. Fractions of 5 ml were collected. Before assays for stimulatory activity were performed, flow through (non-bound material) and bound material (eluate) were pooled together separately, concentrated using 30 kDa filters, washed thrice with PBS (phosphate buffered saline), and then sterilized by filtration through a 0.22-µm syringe filters (Millipore). All procedures were carried out at 4°C.

HiTrap-Q Chromatography

Active concentrated non-binding fraction was equilibrated with ~25-30 ml of buffer C and applied to the HiTrap-Q column. The column was washed with five column volume with buffer C, and then bound material was eluted with 50 ml gradient of a 0.025 - 1 M KCl

gradient at a flow rate of 150 ml/h. Fractions of 5 ml were collected. Before assaying for stimulatory activity fractions were pooled, concentrated and sterilized as described above.

HiPrep 26/60 200 Size Exclusion Chromatography

Active concentrated non-binding fraction was equilibrated with 5-10 ml of buffer D and applied to the HiPrep 26/60 200 size exclusion column (GE). The column was previously equilibrated with buffer D. The gel filtration was carried out at a flow rate of 60 ml/h using column buffer D. Fractions of 5 ml were collected. Proteins were eluted in multiple peaks and pooled according to their UV absorbance profile. Before assaying for their stimulatory activity pooled fractions were concentrated, washed and sterilized as described above.

SDS-PAGE—SDS-PAGE was performed according to the method of Laemmli on 10% polyacrylamide slab gels (1.5 mm thickness). After electrophoresis, gels were either stained using silver staining reagents or Coomassie blue staining. Apparent molecular weight was determined using the protein standards.

Protein Concentration Determination—The protein content of fractions, the peaks and all elution fractions resulting from the columns were quantified using the Bio-Rad® Bradford test, which uses bovine serum albumin (BSA) as a standard. Fractions with low concentrations of protein were assayed at A₂₈₀.

In-Gel Digestion—To dried, gel-bound protein, 200 ng trypsin (modified sequencing grade; Promega, Madison, WI, USA) in 10 µl 100 mM ammonium bicarbonate was added. An additional volume of ammonium bicarbonate was added until the gel piece was covered with the digestion solution; this volume is usually 25–50 µl, depending on the acrylamide volume (surface area) of the original gel piece that was excised for analysis. Tryptic digest was allowed to proceed at 37°C for 2 h. Generated peptides were removed and “cleaned up” using Zip Tips (EMD Millipore, Billerica, MA, USA) as the standard protocol described [24].

NanoLC- MS/MS—Peptides were eluted with 3 µl 40% (v/v) acetonitrile containing 0.1% (v/v) formic acid and diluted to 20 µl with 0.1% formic acid. If not analyzed immediately, the peptide pools are stable when kept in organic buffer (40% acetonitrile/0.1% formic acid) and stored at –80°C. Peptide mixtures were loaded onto a trapping guard column (0.3 × 5 mm Acclaim PepMap 100 C18 cartridge; LC Packings, Sunnyvale, CA, USA) using the Nano MDLC system (Eksigent Technologies, Dublin, CA, USA) at a flow rate 20 µl/min. After washing, the flow was reversed through the guard column and the peptides eluted with a 5–45% acetonitrile gradient over 85 min at a flow rate of 200 nl/min, onto and over a 75 µm × 15 cm-fused silica capillary PepMap 100 C18 column (LC Packings). The eluent was directed to a 75 µm (with 10 µ orifice)-fused silica nano-electrospray needle (New Objective, Woburn, MA, USA). The electrospray ionization needle was set at 1800 V. A linear ion trap quadrupole (LTQ) Orbitrap hybrid analyzer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in automatic, data-dependent MS/MS acquisition mode with 1 MS full scan [*m/z* 450–2000] in the Orbitrap analyzer at 60,000 mass resolution and up to 10 concurrent MS/MS scans in the LTQ for the 10 most intense peaks selected from each survey scan.

Survey scans were acquired in profile mode, and MS/MS scans were acquired in centroid mode. The collision energy was automatically adjusted in accordance with the experimental mass (m/z) value of the precursor ions selected for MS/MS. Minimum ion intensity of 2000 counts was required to trigger an MS/MS spectrum; dynamic exclusion duration was set at 60 s [25].

After nanoLC-MS/MS analysis, database searches in SwissProt 2014 containing 546,000 protein entries were carried out using Mascot (Matrix Science, Boston, MA, USA), version 2.5.00 [26] with the following search parameters: 1) 2 missed cleavage tryptic sites allowed, 2) precursor ion mass tolerance = 10 ppm, and 3) fragment ion mass tolerance = 0.8 Da; variable peptide modifications were allowed for methionine oxidation, deamidation of asparagine to glutamine, protein N-acetylation, and protein and peptide and lysine dimethylation. We carried out additional searches with N-formyl as a modification, as dimethylation and formylation modifications are close in resulting mass difference (8 mass). A decoy database search was always activated, and in general, with $P < 0.01$, a false discovery rate (FDR) averaged $<1\%$.

Database Search—Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.02) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot was set up to search the uniprot_sprot_20130404 database (selected for Homo sapiens, unknown version, 20327 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search a subset of the uniprot_sprot_20130404 database also assuming trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 ppm. Deamidation of asparagine and glutamine, oxidation of methionine, acetyl of the n-terminus and propionamide of cysteine were specified in Mascot as variable modifications. Glu->pyro-Glu of the n-terminus, ammonia-loss of the N-terminus, Gln->pyro-Glu of the n-terminus, deamidated of asparagine and glutamine, oxidation of methionine, acetyl of the N-terminus were specified in X! Tandem as variable modifications.

Criteria For Protein Identification—Scaffold (version Scaffold_4.6.1, Proteome Software Inc., Portland, OR) was used to validate nanoLC-MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 99.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [27]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Results

Growth of ALL3 Cells at Different Starting Densities

We followed the growth of the ALL3 cells at different starting cell densities of 0.5×10^4 , 1×10^4 , 20×10^4 , and 3×10^4 cells/ml using the trypan blue exclusion method. The ALL3 cells were growing progressively faster at higher starting cell densities (HD) of 20×10^4 - 30×10^4 cells/ml (Figure 1A) and they did not grow except very transiently at low starting cell

densities (LD) of $0.5 \times 10^4 - 1 \times 10^4$ cells/ml (Figure 1B). We also found that the cultured filtered supernatant collected from ALL3 cells growing at high density stimulated the growth of the LD ALL3 cells using [^3H]-thymidine incorporation assay (Figure 1C). Thus it suggests that the diffusible, soluble, and secreted factors were able to stimulate the growth of the non-growing LD ALL3 cells, but the ALL3 cells at low starting cell densities failed to grow in the same condition without stimulation.

Comparison of Growth of ALL3 Cells in Serum-Protein Free Media

Next, we decided to use the conventional biochemical purification methods to identify the unknown stimulatory, diffusible, and soluble factor(s) present in the secretome of the HD ALL3 cells. The filtered supernatant used to stimulate the LD ALL3 cells in Figure 1C was collected from the HD ALL3 cells grown in ALL3 media containing 10%FBS. The abundant serum proteins present in the FBS pose major obstacles in the biochemical purification methods and down-stream protein mass spectrometry analysis [28]. Previously, we used affinity chromatography to remove albumin and had some success in removal of albumin but removal of transferrin was still a problem [28]. So, we investigated other serum or protein free media and determined whether the HD ALL3 cells grown in these media were stimulatory enough for the growth of the LD ALL3 cells. We used CellGro media from Mediatech that is totally serum and protein free media. When the HD ALL3 cells were attempted to grow in CellGro, even at high starting cell density of 1.5×10^6 , 3×10^6 , and 3.5×10^6 cells/ml the cells failed to grow (Figure 2A) and most of them died rapidly with approximately 50% of loss in viability in 3 days (Figure 2B). The supernates were collected from CellGro grown HD ALL3 cells after 1 or 2 days of growth. Then the supernates were filtered and concentrated using 5 kDa MWCO filters and >5 kDa fractions were suspended with ALL3 media and kept with the LD ALL3 cells. We did not observe any stimulatory activity of the concentrated supernatant collected from CellGro grown HD ALL3 cells using the [^3H]-thymidine incorporation assay (data not shown). Several other protein-free media such as ExCell SF302 (Sigma Aldrich, St. Louis, MO #24326C) and ExCell SF325 (Sigma Aldrich, St. Louis, MO #14340C) were also tested to see if they would support growth of ALL3 cells at different cell densities, but there was no growth and the cells all died even more rapidly than in Cell Gro.

Comparison of Growth of ALL3 Cells in Serum and/or Protein Free Media

We also used QBSF-60 media from Quality Biological that contains defined amount of recombinant proteins (bovine serum albumin, recombinant human insulin and human transferrin), but are devoid of serum proteins. The ALL3 cells grew very well in QBSF-60 media at higher starting cell densities. We cultured the HD ALL3 cells in QBSF-60 for 3 days and supernates were collected. Then the supernates were filtered, concentrated using the 5 kDa MWCO filters, reconstituted with the 6-7 ml of ALL3 media, and then kept with the LD ALL3 cells to follow stimulatory activity using the [^3H]-thymidine incorporation assay. In parallel, the HD ALL3 cells were also grown in ALL3 media with 10% FBS and concentrated using 5 kDa MWCO filters to compare their stimulatory activity with the QBSF-60 fractions. As expected the ALL3 media based whole high density supernates and concentrated >5 kDa fractions stimulated the growth of the LD ALL3 cells (Figure 2C). The ALL3 media based >5 kDa HDSN concentrated fractions were almost twice more

stimulatory than the whole filtered HDSN on the LD ALL3 cells. As shown in Figure 2D, QBSF-60 based HDSN and concentrated >5 kDa fractions also stimulated the growth of LD ALL3 cells. However the ALL3 media grown HDSN and concentrated fraction were more stimulatory on the LD ALL3 cells (Figure 2C) than the QBSF-60 based HDSN and concentrated fraction (Figure 2D). The stimulation by QBSF-60 based HDSN and >5 kDa concentrated fraction were approximately four times less than the ALL3 media based HDSN and concentrated fractions. We attempted to use the biochemical purification scheme to purify the QBSF-60 based filtered high density supernates collected from the HD ALL3 cells. We conclude that there was no advantage in using the QBSF-60 grown supernatant for biochemical purification, at least in part because QBSF-60 has high amounts of albumin and transferrin which are very difficult to get rid of and obscures low-abundance proteins [28]. In addition, the QBSF-60 media is extremely costly for large-scale culture with multiple trial experiments to optimize the biochemical purification scheme compared to ALL3 media containing 10% FBS.

Comparison of Growth of ALL3 Cells in Serum Reduced Media

Next, we investigated whether reduced amount of FBS in ALL3 media was as stimulatory for the LD ALL3 cells as 10% FBS, rather than using serum or protein free medium which were either very detrimental for ALL3 cells or unreliable for biochemical purification steps. As shown in Figure 3A, the HD ALL3 cells grew equally well in short term culture in 10%, 5%, and 3% FBS containing ALL3 media with excellent viability (~95%) after 3 days of the growth, but they grew very poorly in the presence of 1% FCS and the viability dropped to ~75% by 3 days. We collected supernates from the ALL3 cells (Figure 3A) grown with different amount of FBS in ALL3 media. The supernates were filtered and tested to see how they affect stimulation of the LD ALL3 cells. After collecting high density supernates, all of them were normalized to 10% FCS. As shown in Figure 3B, the HDSN from ALL3 cells grown with 10% FCS were most stimulatory on the LD ALL3 cells than the HDSN collected from the 5% and 3% FBS containing ALL3 media. The HDSN collected from 1% FBS containing ALL3 media was not stimulatory at all to the LD ALL3 cells. Although there was approximately ~50% reduction in the stimulatory activity on the LD ALL3 cells for 5% than 10% FBS grown ALL3 cells' HDSN, we decided to grow the HD ALL3 cells with 5% FBS ALL3 media and use this HDSN for biochemical purifications. The advantages of using 5% FBS ALL3 media to grow the HD ALL3 cells is that the stimulatory profile on the LD ALL3 cells were similar to that of the 10% FBS and low abundant proteins in the HDSN were much easier to detect.

Biochemical Purification Scheme to Identify Active Fraction(s) from HDSN

We decided to grow the HD ALL3 cells in the 5% FBS containing ALL3 media for 3-4 days. The supernates were filtered and then used for biochemical purification. We followed the purification protocol as schematized step wise in Supplementary figure 1A. First, we loaded the 5% FBS containing ALL3 media based HDSN ALL3 supernatant on a HiTrap-Q column and washed it with buffer A, and then eluted the bound material with buffer B (see procedure in method section for more detail). The flowthrough (non-binding material) and eluate (binding material) were collected and concentrated using 30 kDa MWCO filters, and their stimulatory activity on the LD ALL3 cells was determined using the [³H]-thymidine

incorporation assay. The fractions were also run on the SDS-PAGE to determine the performance of the purification step. We found that all the stimulatory activity was present in the flow-through fraction. In the second step, we resuspended the active flow-through fractions from step 1 with buffer C, and then loaded it again onto the HiTrap-Q column. The same procedure was repeated as described above for the first step. We also found that all the stimulatory activity was present in the non-binding concentrated fraction from step 2 (see step 2 in supplementary Fig 1 for more detail). In the third step, the concentrated non-binding fraction from the second step was resuspended with buffer D, and then loaded on to HiPrep 26/60 200 size exclusion column (see step 3 in supplementary Fig1 for more detail). The fractions were pooled together according to their UV absorbance peak profile, and then concentrated using 30 kDa MWCO. The concentrated fractions were tested individually to test their stimulatory activity on the LD ALL3 cells using the [³H]-thymidine incorporation assay. The stimulatory activity of each fraction using the [³H]-thymidine incorporation assay is shown later in this section.

As shown in supplementary figures 1B the majority of the serum proteins were bound to the column (represented as B1st) from step 1. After step 1, the active fraction (represented as NB1st in supplementary figure 1B) was reduced in contaminating high abundance FBS proteins. Further on the second step the non-binding active fraction (represented as NB2nd in supplementary figure 1B) was enriched for other lower abundant proteins and reduced in contaminating inactive binding fractions proteins (represented as B 2nd in supplementary figure 1B). Use of the second step HiTrap-Q was very beneficial to get rid of the majority of remaining highly abundant serum protein(s) and the non-binding fraction was found to be enriched for less abundant proteins as can be visualized by silver stained gel lanes comparing the non-binding fractions from the second (NB2nd) and first step (NB1st).

Next, the active non-binding fraction from the second step was loaded on the size exclusion column (refer to step 3 in method section for more detail). The fractions were pooled together, concentrated, and tested for their stimulatory activity on the LD ALL3 cells using the [³H]-thymidine incorporation assay. The concentrated fractions were also run on the gel and visualized using the silver staining method. As shown in supplementary figure 1C, the fractions became more highly enriched for lower abundant proteins after the size exclusion chromatography.

We followed the activity of the fractions after each biochemical purification steps using the [³H]-thymidine incorporation assay. The effect of the concentrated fractions collected after each biochemical purification steps compared to the starting material were tested to determine their effect on the LD ALL3 cells using the [³H]-thymidine incorporation assay. Once the activity of the fractions was confirmed, the active fraction(s) were further subjected to the next step to get a purer enriched active fraction. As shown in Figure 4A, after step 1 HiTrap-Q all the stimulatory activity was present in the concentrated non-binding fraction. After second step HiTrap-Q we also found that the stimulatory activity was present in the non-binding fraction. In the third step size exclusion chromatography fraction2 (arbitrary numbered) was the most active on the LD ALL3 cells (supplementary figures 1C and Figure 4C). The active non-binding fraction from step 1 was showing almost similar stimulatory profile to that of the whole HDSN. However, the whole HDSN was maximally stimulated

the LD ALL3 cells on day 11 while the non-binding concentrated fraction gave maximum stimulation around day 15 (Figure 4A). The non-binding concentrated active fraction from step 2 was more stimulatory around ~17-24 days (Figure 4B). As expected the whole HDSN stimulated the LD ALL3 cells maximally Day ~9, but from the concentrated fractions fraction 2 (represented as Fx2>30 kDa) was the most stimulatory fraction and it gave maximum stimulation around day ~20 in step 3 (Figure 4C). It is important to note that the LD ALL3 cells without HDSN grew little bit after 2 weeks of growth in culture (Figure 4C), but it does not change final conclusion.

The overall purification procedure led to a concentration of the stimulatory factor(s) is shown in Table 1. The active fractions were ~500× more enriched for stimulatory factor(s) as shown using purification factor accompanied with ~100× increase in specific activity (average counts per minute/per total protein). However, as visualized using silver stained gel lane (Supplementary Figure 1C, Fx2 lane) there were still lots of proteins in that fractions so a few more steps are required to obtain more pure and enriched active fraction.

NanoLC-MS/MS analysis of the stimulatory factor(s)

To identify the proteins responsible for the stimulatory activity, we also subjected all the fractions (1 to 6; designated as A to E for nanoLC-MS/MS) to nanoLC-MS/MS analysis (Supplementary figure 2). The nanoLC-MS/MS confirms that the albumin and transferrin were almost removed from all the fractions (shown as spectral counts). The list of proteins obtained from nanoLC-MS/MS are given in Table 2 A to F. Additional information of the proteins identified by nanoLC-MS/MS, i.e., number of unique peptides (supplementary table 1), number of unique spectra (supplementary table 2), number of total spectra (supplementary table 3), peptides per protein and the amino acid sequences of each peptide (supplementary table 4) are shown in supplemental tables.

In our nanoLC-MS/MS experiments, we identified 79 proteins (identified by two or more peptides). However, we found that not all the proteins identified in the nanoLC-MS/MS analyses were secreted proteins, mostly because contamination by non-secreted proteins due to, for example, to cell death or cell apoptosis was unavoidable. The protein list was subjected to various bioinformatics analysis to determine their cellular component. We used PANTHER [29] (supplementary table 5), EnrichR [30, 31] (supplementary table 6) and Scaffold (V4.6.1) (supplementary table 7) (Supplementary Figure 3) to find their cellular locations. From these proteins, several were intracellular proteins (i.e. housekeeping proteins such as Alpha-enolase, Triosephosphate isomerase, Malate dehydrogenase, cytoplasmic) and could be eliminated as possible stimulatory factor candidates. Based on this analysis out of 79, only 26 proteins were chosen for further analysis. So, since we expected the stimulatory factors to be somehow secreted, but did not know the exact mechanism of secretion, we investigated the proteins identified in our nanoLC-MS/MS experiments by bioinformatics analysis. We used SignalP [32] to identify the proteins that contain signal peptides and are secreted through the classical secretory pathway and SecretomeP [33] to identify the proteins that have no N-terminal signal peptide, but that can still be secreted via non-classical secretory pathway. This information is indicated in Table 2. Out of 26 proteins, 8 proteins were found to be secreted either by classical or non-classical secretory pathways.

We would also like to note that, in addition to being secreted by classical or non-classical secretory pathways, the proteins are also released through the exosomes [9].

Possible Stimulatory factor candidates

From the entire list of proteins found in nanoLC-MS/MS analysis, after eliminating the intracellular, housekeeping proteins, here we discuss the function of 8 most promising candidates that qualify as stimulatory factors. The secretory candidates include AHNAK, DCD, DSG1, ERO1A, GSN, LCN1, SEMG1, and ZA2G (AZGP1) (Table 2).

AHNAK, also known as neuroblast differentiation-associated protein, is involved in mesothelioma [34], head and neck cancer [35], and laryngeal carcinoma [36]. It was found that AHNAK is also involved in regulating the production and release of exosomes [37] from mammary carcinoma cells and also control motility of fibroblasts. AHNAK knockout mice showed increase in hippocampal neuronal proliferation [38].

DCD gene encodes a 110-amino acid polypeptide. The polypeptide contains a 20 amino acid signal peptide, the 30 amino acid proteolysis inducing factor-core peptide (PIF-CP), a 13 amino acid propeptide and the 47 amino acid DCD-1 peptide. DCD induces cell proliferation in hepatic cells [39] and improves cell survival [40, 41]. DCD-1 peptide and PIF-CP are proliferation and survival factors in several cancers [40, 42, 43].

DSG1 is involved in pancreatic ductal adenocarcinoma [44], lung cancer [45], oral neoplasms [46] and carcinoma [47], prostate cancer [48], anal carcinoma [49], head and neck squamous cell carcinoma [50], and skin squamous cell carcinomas [51, 52]. DSG1 is a transmembrane glycoprotein. It is involved in regulating keratinocyte differentiation [53, 54], epidermal differentiation [55, 56] & morphogenesis [55].

ERO1- α (endoplasmic reticulum disulfide oxidase) is involved in formation of disulfide bonds which are required for the proper conformation and function of secreted proteins [57]. ERO1- α is found to be overexpressed in gastrointestinal cancer [58], and breast cancer [59]. Gelsolin (GSN) regulates cell proliferation, invasion, apoptosis & migration [60-62]. LCN1 is a family of small secretory proteins that binds to hydrophobic ligands [63]. AZGP1 is a glycoprotein. It is associated with the cachexia related to cancer [64, 65]. It is involved in prostate cancer [66-68], colon cancer [69, 70], breast carcinoma, gastric cancer [71], and hepatocellular carcinoma [72].

Discussion

Direct cell-to-cell contact, soluble secretory proteins, gap junctions, and shedding or release of extracellular vesicles are involved in intercellular communications. Secretome proteins have been implicated in various processes such as proliferation, apoptosis, invasion, metastasis, cell patterning, and cell-cell communication.

The Ph⁺ ALL3 cell line was used as a model system to try to understand how cells communicate with each other and with other cells and the abnormalities in quorum sensing that permit these S/P cells to far exceed the normal homeostatic cell densities without

addition of any of the known cytokines or other growth factors. The ALL3 cells do not grow at low starting cell densities but grow very well with good viability at high starting cell densities. This observation is nicely coordinated with older observations in bacteria in which light emission was determined to occur only at a high cell population density [73].

One of the widely used methods to isolate and identify the unknown stimulatory factor(s) is to adopt a sequential biochemical purification step. Successful identification of unknown stimulatory factor(s) requires three steps. First, reliable source of stimulation could be primary cells, cell lines or tissues. Second, to observe the stimulation or any functional activity it is necessary to employ an accurate quantitative assay system sensitive enough to measure significant functional changes in relatively small number of cells. Third, a purification scheme which enables one to exclude non-stimulating and/or contaminating proteins.

Fetal bovine serum (FBS) is routinely used as a supplement to grow most of the cell lines and primary cell because it facilitates the cell growth. Serum deprivation/starvation has been used intensively as a model of cellular stress [74-76]. Serum deprivation in the culture media removes known or unknown contaminants, provide experimental flexibility, reduces downstream analytical interference (such as LC-MS/MS analysis), and avoid complex upstream biochemical purification step, but it induces several unwanted biochemical changes like increase in apoptosis, change in phosphorylation of several proteins, alterations in composition of microvesicles and many more [74-77]. Undoubtedly, serum starvation is an important model system to study several cellular mechanisms. In our study, we found that serum starvation or serum reduction affected fold response to various stimuli which might be due to change in cellular phenotypic characteristics.

Our current experimental system allowed us to advance our understanding of quorum sensing and communication between the cells, depending on their density. We found that LD cells can be stimulated by one or more stimulatory factor(s) secreted either as soluble factors or in exosomes in the secretome of proliferating HD ALL3 cells [8, 9]. We were also able to show that using nanoLC-MS/MS analysis and then bioinformatics analysis that it was possible to identify several proteins in the purified fractions that had stimulatory activity, which could be candidates but which of course require verification that when purified alone or in combination they are indeed stimulatory.

Conclusion

In this article we have used biochemical purification methods to identify the possible protein candidates involved in growth of non-growing LD ALL3 cells. The availability of ALL3 cells line, biochemical purification steps, and protein candidates will enable direct examination of quorum sensing behavior in various cell types. In additions, it may allow exploration of the clinical potential of such a stimulatory molecule. Lastly, we hope that the concepts and methods developed in the study of quorum sensing of ALL3 will be applicable to other systems whose molecular cues are still unknown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALL	Acute lymphoblastic leukemia
QS	Quorum sensing
HD	High starting cell density
LD	Low starting cell density
HDSN	High density supernates

Figure 1.

Growth of the ALL3 cells at different conditions.

The ALL3 cells were grown in the ALL3 media as described in 'Methods'. Comparative growth of the ALL3 cells at starting high cell density (HD) of A) 20×10^4 and 30×10^4 cells/ml and low starting cell density of B) 0.5×10^4 - 1×10^4 cells/ml. Y-axis represents total number of viable cells on different days determined using trypan-blue exclusion method. Note: In both cases, A) and B) Y-axis are of different scale. C) The [³H]-Thymidine uptake assays showing the stimulatory effect of HDSN collected from HD ALL3 cells on the growth of LD ALL3 cells (red bar) to that of LD ALL3 cells without stimulation (blue bar). The y-axis represents the counts per minute (CPM). Values are mean $\pm$ SD for triplicates from a representative experiment.

Figure 2.

Growth of the ALL3 cells in different media.

A) The ALL3 cells were grown in CellGro medium and the growth was followed using trypan-blue dye exclusion method. Comparative growth of ALL3 cells in CellGro at starting high cell density of 1.5×10^6 , 3×10^6 , and 3.5×10^6 cells/ml. Y-axis represents total number of viable cells on different days. B) Viability of ALL3 cells grown in A), shows percentage of live cells at different days.

C)&D) show the [³H]-Thymidine uptake assays showing the stimulatory effect of HDSN collected from HD ALL3 cells grown in presence of C) ALL3 media and D) QBSF-60 on the growth of LD ALL3 cells. The HDSNs were collected from ALL3 cells grown with both medias and also concentrated using 5 kDa MWCO filter. Blue bar: LD ALL3 cells, red bar: LD ALL3 cells with HDSN, green bar: LD ALL3 cells with concentrated >5 kDa HDSN, and magenta bar: LD ALL3 cells with concentrated <5 kDa HDSN. In both cases, C) and D) the y-axis represents the counts per minute (CPM). Values are mean $\pm$ SD for triplicates from a representative experiment.

Figure 3.

Growth of the ALL3 cells in presence of different concentrations of FBS in ALL3 media. The ALL3 cells were grown in ALL3 medium containing different concentration of FBS as described in 'Methods'. A) Comparative growth of ALL3 cells at starting cell density of 5×10^5 cells/ml containing different concentration of FBS in ALL3 media as shown. The percentages of the viable cells are shown in bracket. Y-axis represents total number of viable cells on different days as determined using trypan-blue exclusion method. B) The ^3H -Thymidine uptake assay showing the stimulatory effect of HDSN collected from the HD ALL3 cells growing with different concentration of FBS in ALL3 media on the growth of LD ALL3 cells. The amount of FBS was normalized to 10% FBS after collecting the HDSN. The y-axis represents the counts per minute (CPM). Values are mean $\pm$ SD for triplicates from a representative experiment.

Figure 4.

Stimulatory activity of the LD ALL3 cells in presence of different fractions collected from purification scheme using $[^3\text{H}]$ -Thymidine uptake assays for A) Step-1 HiTrap-Q method, B) Step-2 HiTrap-Q method, and C) Step-3 HiPrep 26/60 200 size exclusion column. The y-axis represents the counts per minute (CPM). The fractions are concentrated using 30 kDa MWCO filters, then washed two-three times with PBS and suspended with ALL3 media to stimulate the LD ALL3 cells.

Table 1
Purification of Stimulatory Factor From Cell Culture Supernatant Collected From ALL3 Cells at High Density.

	Volume	Total Protein	Activity (CPM)	Specific Activity (CPM/mg of protein)	Purification Factor	Recovery
HDSN	500 ml	6670 mg	88.75	13.33×10^3	1	100%
SEC	0.5 ml	4.3 mg	6.54	1495.9×10^3 (112.4X)	525.6	7.4%

Table 2

List of proteins subjected to SecretomeP and SignalP 4.1 to identify secretory proteins.

	SignalP 4.1	SecretomeP 2.0
AHNK (AHNAK)	NO	Yes
ANXA1	NO	NO
ANXA2	NO	NO
ARGI1	NO	NO
CALL3	NO	NO
CALL5	NO	NO
CALM	NO	NO
CASPC	NO	NO
CASPE	NO	NO
CATA	NO	NO
DCD	Yes	NO
DESP	NO	NO
DSG1	Yes	NO
EF1A1	NO	NO
ERO1A	Yes	Yes
GELS (GSN)	NO	Yes
HORN	NO	NO
LCN1	Yes	NO
PRDX1	NO	NO
PRDX6	NO	NO
QOR	NO	NO
S10A9	NO	NO
SEMG1	Yes	NO
SPB3	NO	NO
SPB4	NO	NO
ZA2G (AZGP1)	Yes	Yes