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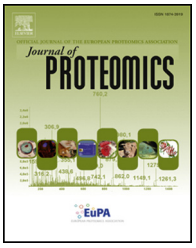

Interleukin-1-induced changes in the glioblastoma secretome suggest its role in tumor progression

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ABSTRACT

The tumor microenvironment including glial cells and their inflammatory products regulates brain tumor development and progression. We have previously established that human glioma cells are exquisitely sensitive to IL-1 stimulation leading us to undertake a comparative analysis of the secretome of unstimulated and cytokine (IL-1)-stimulated glioblastoma cells. We performed label-free quantitative proteomic analysis and detected 190 proteins which included cytokines, chemokines, growth factors, proteases, cell adhesion molecules, extracellular matrix (ECM) and related proteins. Measuring area under the curve (AUC) of peptides for quantitation, the IL-1-induced secretome contained 13 upregulated and 5 downregulated extracellular proteins ($p < 0.05$) compared to controls. Of these, IL-8, CCL2, TNC, Gal-1 and PTX3 were validated as upregulated and SERPINE1, STC2, CTGF and COL4A2 were validated as downregulated factors by immunochemical methods. A major representation of the ECM and related proteins in the glioblastoma secretome and their modulation by IL-1 suggested that IL-1 induces its effect in part by altering TGFβ expression, activity and signaling. These findings enhance our understanding of IL-1-induced modulation of glioma microenvironment, with implications for increased tumor invasion, migration and angiogenesis. They further provide novel targets for the glioblastoma intervention.

Biological significance
Present study is on an unbiased screening of the glioblastoma secretome stimulated by IL-1 which triggers neuroinflammatory cascades in the central nervous system. Network of secreted proteins were shown to be regulated revealing their possible contribution to glioma progression. Label free quantitative proteomics has provided unique novel targets for potential glioblastoma intervention.

1. Introduction

Glioblastoma multiforme (GBM) is the most common brain tumor with very poor prognosis despite recent progress in chemotherapy and immunotherapy [1–3]. The poor prognosis of malignant gliomas is related to the ability of tumor cells to infiltrate the surrounding tissue, making the tumor non-resectable, as well as their high degree of neo-angiogenesis

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and tumor necrosis [4]. During tumorigenesis/progression, the normal balance between critical proteins such as proteases, their inhibitors, and adhesion molecules is dysregulated. It is also expected that other not yet identified proteins are involved in this process. In addition to the cell-associated proteins, glioma-produced secreted proteins also play a major role in cell-cell communication with other glioma cells, tumor vessels, inflammatory cells, and endogenous brain cells [3]. Therefore, analysis of proteins secreted by glioblastoma cells in an unbiased manner (secretome) is an important approach to understand these mechanisms.

Gliomas in vivo grow in a highly complex microenvironment. Microglia and astrocytes are among the brain cells implicated in facilitating glioma growth, invasion, angiogenesis and anti-tumor surveillance [3,5]. Tumor-associated microglia and astrocytes show an activated phenotype with a global change in their transcriptional, metabolic and secretory profiles, but the details of these changes and their impact on glioma progression are not known. Of the soluble mediators that are produced by activated microglia, IL-1 plays a particularly important role in the activation of glioma cells and initiating the neuroinflammatory cascade in the central nervous system (CNS) [6–9]. Human glioblastoma cells are exquisitely sensitive to IL-1 stimulation [10] (*and this report*). Importantly, evidence also supports that glioblastoma cells in vivo and in vitro also produce IL-1 [11,12] (*Tarassishin and Lee, unpublished*) and that IL-1 itself is also a potent inducer of IL-1 production in myeloid cells and in human glioma cells [13,14] (*manuscript in preparation*). These results together indicate that IL-1-mediated cellular interactions involving GBM cells, tumor-associated microglia, neuroglial cells, and blood vessels can set off a potent proinflammatory, pro-tumor and neurotoxic cascade in the CNS [15–17].

Given the importance of inflammation in tumorigenesis and progression [18,19], and the central importance of IL-1 in the establishment of glioma microenvironment, in the present study we performed quantitative proteomic analysis of the glioblastoma secretome, comparing unstimulated and IL-1-stimulated glioblastoma cells (U251). This approach provides a platform of non-targeted and unbiased discovery for large number of differentially secreted proteins. We found 190 proteins including cytokines, growth factors, ECM and related proteins. Their altered abundance upon IL-1β stimulation imitates the inflammatory response and provides insight into the extracellular events resulting in pro-tumor environment.

2. Materials and methods

2.1. Cells, reagents, and sample preparation

Glioblastoma cell line U251 was cultivated in DMEM supplemented with 5% FBS and antibiotics ("Anti-anti" from Invitrogen/Life Technologies, Grand Island, NY). Recombinant human IL-1β was purchased from Peprotech (Rocky Hill, NJ). For cytokine stimulation, the cells were grown in 25 cm dishes until ~ 90% confluence, then incubated with IL-1β (10 ng/ml) for 15 min, which we previously determined to be sufficient to activate human astrocytes [20]. Cultures were then washed extensively with PBS to remove carry over cytokines and were further incubated with serum-free DMEM for an additional 24 h. Control medium was produced similarly except that the cytokine treatment was omitted.

Conditioned medium (CM) was collected by gentle aspiration and then centrifuged at 2000 rpm for 10 min to remove cell debris. CM was concentrated using polyethersulfonate membrane > 5000 MWCO (Sartorius Stedim Biotech GmBH, Gethingen, Germany), and washed twice with sterile ddH_20 (×2 volume) in order to decrease the salt loading at MS. Samples were then denatured by boiling for 5 min and centrifuged at 10,000 rpm for 5 min to remove possible protein aggregates. Protein concentration was determined with Bio-Rad Protein Assay reagents (Bio-Rad, Hercules, CA). This process was repeated three times to collect three sets of controls and stimulated media.

2.2. SDS-PAGE and Western blotting

Ten (10) micrograms of samples was incubated with equal volume of 2× dissociation buffer for 5 min at 95 °C and applied to the Criterion 4–20% gradient gel or 10% gel as indicated. After electrophoresis, the gels were stained with Bio-Safe Coomassie Blue. All reagents were from Bio-Rad.

For Western blotting, the proteins were transferred to polyvinylidene difluoride membrane. The membrane was blocked in PBS containing 5% nonfat milk and then incubated with antibodies at 4 °C for 16 h. Primary antibodies were: anti-Tenascin-C, anti-Galectin-1, and anti-Pentraxin 3 (1:1000, R&D Systems, Minneapolis, MN), anti-Stanniocalcin-2 (1:100, ThermoScientific, Franklin, MA), anti-SERPINE1 (1:400, Sigma-Aldrich, St Louis, MO), anti-MMP2 (1:250, Cell Signaling, Beverly, MA), anti-CTGF (1:200, Santa Cruz Biotech, Santa Cruz, CA), and anti-COL4A2 (1:200, Santa Cruz Biotech). Secondary antibodies were: horseradish peroxidase (HRP)-conjugated anti-goat IgG (1:5000, Rockland Immunochemicals, Gilbertville, PA), anti-rabbit IgG (1:500) and anti-mouse IgG (1:500, ThermoScientific) and incubated for 1 h at room temperature. Signals were developed using enhanced chemiluminescence (Super Signal West or Pico Chemiluminescent Substrate, ThermoScientific). After developing, the X-ray films were scanned and densitometry analyses were performed with NIH Image J software. Statistical analyses of Western blot data were performed by Student's *t*-test (Ctr vs. IL-1treated samples, n = 3) using GraphPad Prism 5.0.

2.3. ELISA

ELISAs for IL-8, CCL2 and TGFβ1 were performed using R&D Systems DuoSet reagents following the manufacturer's protocols. The sensitivity ranges for ELISA were 32.2–2000 pg/ml for IL-8, 15.6–1000 pg/ml for CCL2, and 31.2–2000 pg/ml for TGFβ1. The samples were diluted until the values fell within the linear range of detection. Statistical analyses of ELISA data were performed by Student's *t*-test (Ctr vs. IL-1treated samples, n = 3) using GraphPad Prism 5.0.

2.4. Mass spectrometry analysis of conditioned media

Concentrated and washed CM retentates from the membrane filters were loaded on a PAGE gel (12%) and run to ~6 mm to

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