Differential Cancer Biomarker Secretion of Cultured Cells

Use of the ELx50™ to Automate the Wash Steps of Multiplexed Luminex® xMAP® Bead Assays

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Diagnostic biomarkers are an important area of investigation in cancer research today. While historically intercellular biomarkers have been studied, work understanding soluble secreted biomarkers have become more important. Using a 22-plex Cancer Biomarker Assay Panel from EMD Millipore, we characterized the secretion of cancer biomarkers by several human tissue culture cell lines under normal and low serum conditions.

Introduction

Diagnostic biomarkers are a key element in cancer research. Research has focused primarily on intracellular biomarkers, such as HER2 or BRAC1, that identify specific tumors, or provide genetic or phenotypic information that can clarify the process of oncogenesis. Recently more attention is being placed on identifying soluble extracellular circulating biomarkers, which can provide information on the body's response to cancer, as well as the relationship between a tumor cell and its environment. Because cancer is a series of different disease states, the study of individual biomarkers is usually inadequate to study the complex relationship between a tumor and its environment. For example, a panel of four known biomarkers, leptin, prolactin, ostepontin, and insulin like-growth factor II, achieved a predictive value of 95% for the diagnosis of ovarian cancer when used together, but had no predictive value when used alone [1]. While some biomarkers are tumor specific, such as PSA, others such as IL-8, are found in tumors of many different origins.

Using a panel of known tumor biomarkers to characterize tumor cell lines of known lineage under different conditions provides a better understanding of the biology specific to different tumor types. Many different cell types including tumor cells secrete cellular messengers than can influence growth via receptor mediated signaling. If the cell in question secretes a compound that will interact with its own receptors, this is referred to as autocrine secretion. If the secreting messenger interacts with adjacent cells, this phenomenon is referred to a paracrine secretion (Figure 1). Endocrine secretion involves the flow of the messenger signals through capillary vessels and transportation to remotely located cells. In culture only autocrine and paracrine secretion are possible.

The assay is based on Luminex® xMAP® technology. Distinct internally color-coded magnetic microspheres each coated with a specific antibody capture and quantitate different analytes. By using multiplexed bead sets, several analytes can be quantitated simultaneously.

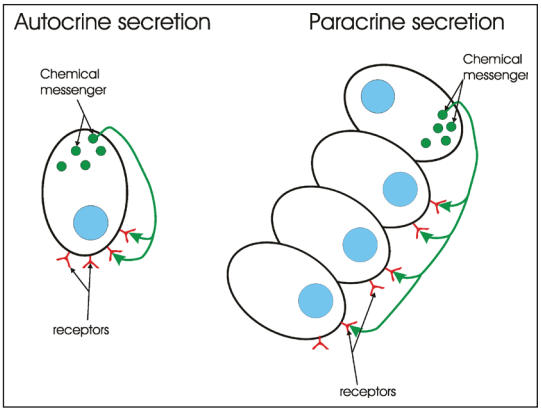

Figure 1. Autocrine and paracrine secretion.

Materials and Methods

The human tissue culture cell lines HepG2, HT1080, and HEK293 were obtained from ATCC. LP9, H-Meso, GA and MI cells were provided by the Vermont Cancer Center at the University of Vermont. DMEM, F12, MEM, F12/DMEM growth media, glutamine and fetal bovine serum (FBS) were obtained from Invitrogen. Hydrocortisone (Cat # H0135) and ITS (cat # I1884) were from Sigma-Aldrich. MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panels were obtained from EMD Millipore. HepG2 and HT1080 cells were maintained in MEM supplemented with 10% FBS and 2 mM glutamine. HEK 293 cells were cultured in DMEM supplemented with 10% FBS.

LP9, H-Meso, GA and MI cell lines were maintained in DMEM/F12 supplemented with 10% FBS, 2 mM glutamine, hydrocortisone 100 ng/mL, and ITS. Aliquots (1.5 mL) of conditioned media were obtained from cultures in log phase growth in the presence of 0.25% or 10% FBS and centrifuged at 14,000 RPM in an Eppendorf 5415C microfuge to remove any particulates.

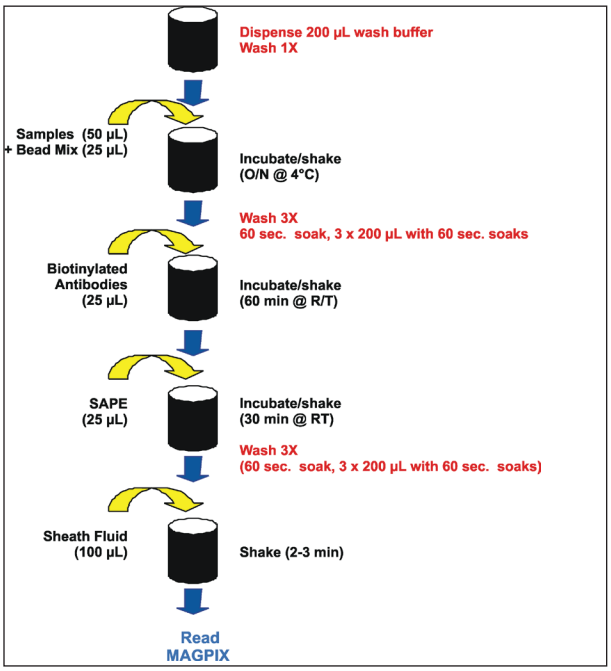

Figure 2. EMD Millipore cancer biomarker assay process. Red text indicates automated processes carried out by the ELx50 Automated Microplate Strip Washer.

The MILLIPLEX assay was performed according to the kit instructions (Figure 2). The assay plate was first washed one time using the supplied assay wash buffer to remove any residue. Eight working multiplex standards were generated by serial dilution (1:3) of the reconstituted human Circulating Cancer Biomarker Panel, which contained 22 different analytes. After reconstitution, 50 µL each of standards and samples were pipetted into bead containing wells of the assay microplate. In parallel, the bead master mix was prepared by combining 150 µL of each individual bead suspension. 25 µL aliquots of the master mix were added to each well, and the reactions were allowed to incubate overnight at 4°C with agitation on a plate shaker. The following day the plate was washed 3 times as previously described [2]. After washing, 25 µL of detection or secondary antibody reagent was added and allowed to incubate for 60 minutes at RT with agitation. The beads were again washed three times followed by the addition of 25 µL of SAPE reagent. After 30-minute incubation with agitation to allow for reporter tag binding to occur, the plate was again washed as described in the washing instructions. The samples and standards were then resuspended in 100 µL of sheath fluid. Samples were then read on either a Luminex MAGPIX® or a 100 (LX100) reader with XPONENT software using the parameters outlined in the assay kit instructions.

The ELx50 Microplate Strip Washer (BioTek Instruments) used in these experiments was configured with a 96-well flat magnet (P/N 7103016). Wash programs were configured via the keypad using the "Link" utility to join three separate routines as described previously [9]. An initial 60 second soak allows for bead capture, while a stepped aspiration scheme reduces bead loss from aspiration.

Results

When human cell lines are tested for cancer biomarker secretion, very different patterns of expression are observed. All of the established cell lines tested secreted IL-8 and MIF, both markers for inflammation. HepG2 cells secreted the greatest number of different tumor markers of the cell lines tested. HepG2 cells were observed to secrete large amounts of AFP when cultured with or without serum. In addition to AFP, HepG2 cells also secrete CYFRA21-1, MIF, OPN and VEGF. Interestingly, CYFRA21-1 and IL-8 were secreted in larger amounts when 10% serum was present. (Figure 3)

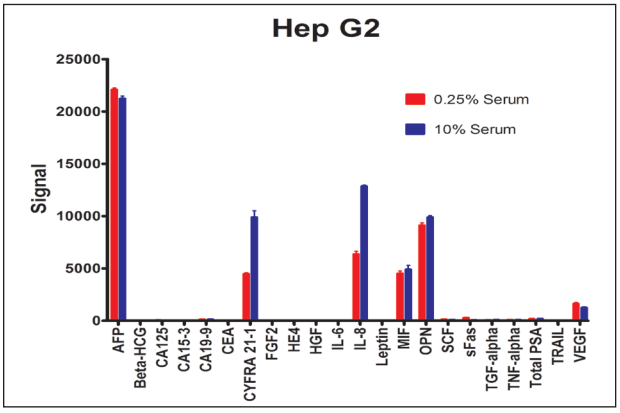

Figure 3. Cancer biomarker 22-plex assay on HepG2 cells.

HT1080 cells also secreted a number of different molecules including large amounts of IL-8. Besides IL-8, these cells also secreted measurable amounts of ß-HCG, FGF2, MIF, OPN, TGF-ß and VEGF. A number of these molecules show an increase in signal as a result of serum, particularly IL-8, VEGF and MIF.

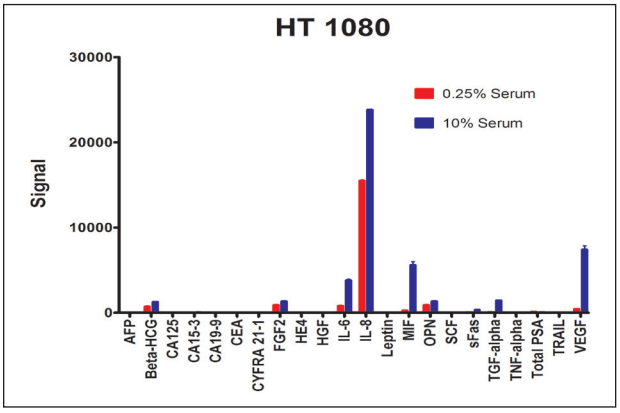

Figure 4. Cancer biomarker 22-plex assay on HT 1080 cells.

Only IL-8 and MIF cancer markers were to be found to be secreted in any abundance when HEK293 cells were characterized using the MILLIPLEX Panel. Unlike the other cell lines tested, IL-8 showed much higher levels in the absence of serum rather than the presence of 10% FBS. MIF was secreted at high levels with or without serum. Low levels of TNF-∞ were also detected.

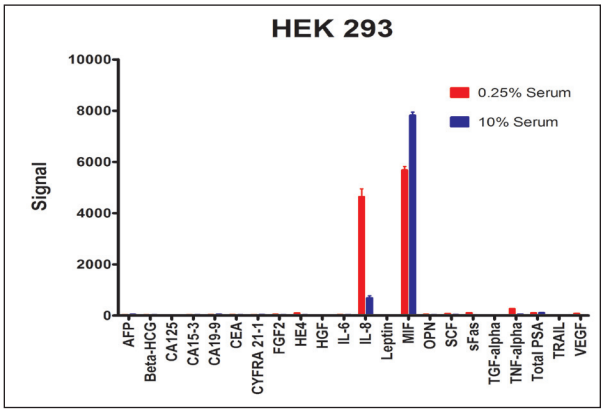

Figure 5. Cancer biomarker 22-plex assay on HEK 293 cells.

LP9 mesothelial cells exhibit secretion of IL-6, IL-8 and MIF markers, with IL-8 being in the greatest abundance. All three markers demonstrate an increase in signal in the presence of 10% FBS as compared to the 0.25% serum samples.

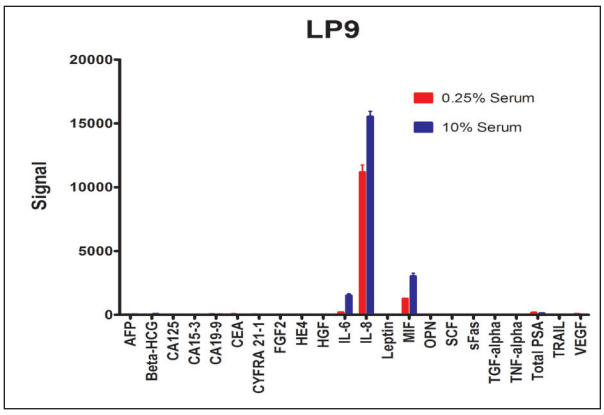

Figure 6. Cancer biomarker 22-plex assay on LP9 cells.

Three different malignant mesothelioma cell lines were characterized. A number of commonalities between the cell lines were observed. Like the other cell lines tested, the mesothelioma cells secrete IL-8 and MIF. They, like the mesothelial cell line LP9, also produce IL-6, suggesting that mesothelial derived cell lines secrete IL-6. Interestingly the GA and MI cell lines also secrete CYFRA21-1, while the H-Meso cell line does not. GA and MI cell lines have only been isolated relatively recently as compared to H-Meso cell line. As with the more undifferentiated established lines (i.e. HEK293), the mesothelioma cell lines respond to low serum by producing more of some markers. H-Meso and GA cell lines secrete more IL-6 and IL-8 in response to low serum, while MI produces more CYFRA21-1. H-Meso and GA cells produce significantly more MIF in high serum as compared to when treated with 10% serum. Low serum also induced the secretion of FGF2 in MI cells (Figure 7).

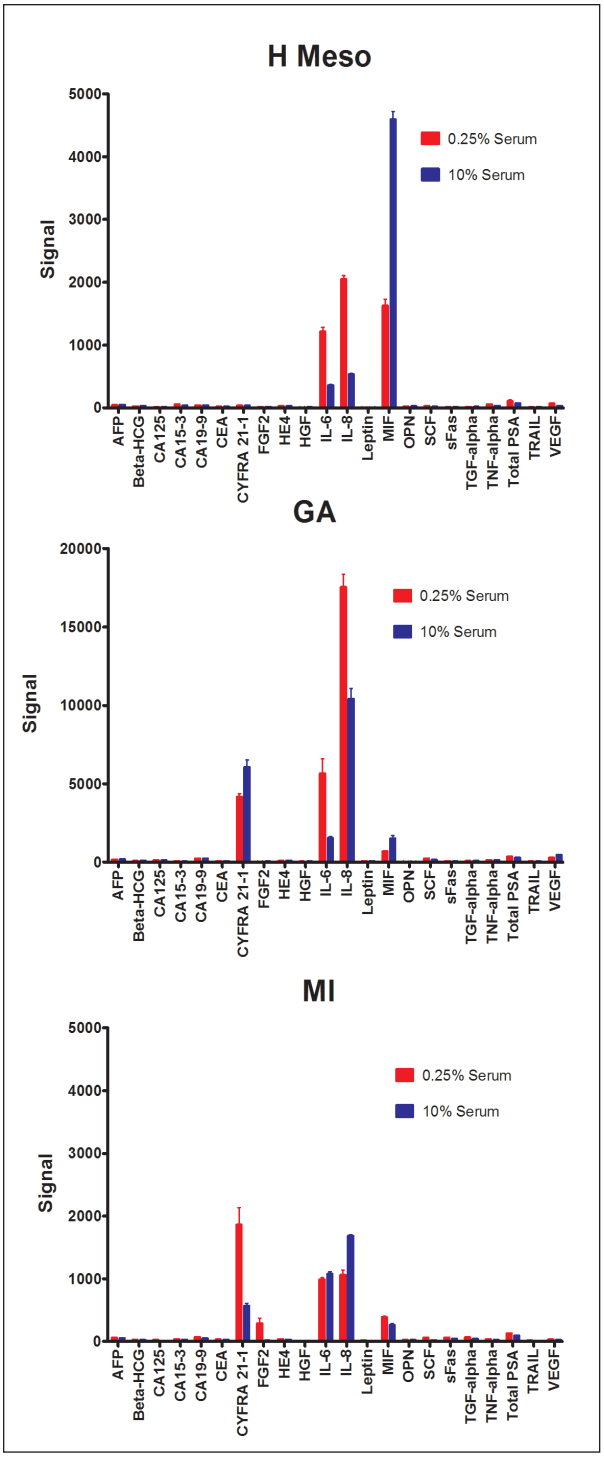

Figure 7. Cancer biomarker 22-plex assay of mesothelioma cell lines.

Discussion

The cell lines characterized showed markedly different phenotypes in regards to secreted cancer markers. In general only a few of the 22 cancer markers tested were secreted by the cell lines tested. The secretion of IL-8 was the most consistent across all the cell lines. Interleukin-8 is a chemokine produced by a number of different cell types. It interacts with the CXCR1 and CXCR2 receptors, which are GPCRs. While is primary function has been described as a chemo-attractant and a mediator of inflammation, it has also been shown to promote cell proliferation [3]. Interestingly while five of the cell lines increased secretion of IL-8 with 10% serum, HEK293 and GA cells actually decreased the amount found in the media. MIF, which was also found to be secreted to some extent by all cell types, increased with high serum in most cell lines. The MI cell line, which produced low amounts of MIF, showed a small decrease. MIF, first identified as a key element of inflammation has also been shown to have a role in tumor progression and cell proliferation [4]. The connection between inflammation and cell growth has been observed to be a factor in tumor promotion.

The observation that HepG2 cells secrete AFP is not surprising. The ∞-Fetoprotein glycoprotein (AFP) is a major plasma protein produced by the yolk sac and the liver during fetal life and is thought to be the fetal equivalent to serum albumin. While at birth infants have high levels of this marker, AFP drops to almost undetectable levels during the first year of life. In adults, elevated levels are only seen in three conditions: hepatocellular carcinoma, germ cell tumors of the testes or ovaries, and metastatic cancer originating from tumors in the liver. HepG2 cells are derived from the liver tissue of a 15 year old Caucasian American male with a well differentiated hepatocellular carcinoma. These cells are also known to secrete plasma proteins such as albumin, transferring, fibrinogen, ∞2-macroglobulin, and plasminogen. As these cells are derived from a well differentiated liver tumor, one would expect that they would secrete AFP.

Human Embryonic Kidney 293 (HEK293) cells are a cell line generated in the early 1970s by transformation of normal embryonic kidney cells with adenovirus [5]. While originally thought to be fibroblasts or epithelial in nature, recent evidence suggests that they may be neuronal in nature [6]. This ambiguity makes them a poor model for either normal or cancer cells, but their ease of culturing and transfection makes them a favorite for experiments where the type of cell is not fundamental to the experiment. The increase in IL-8 secretion by HEK293 is an interesting phenomenon. Increases in many of the tumor markers can be explained as a response to the growth factors in the fetal bovine serum stimulating cell growth. The marked increase in IL-8 secretion may be an autocrine and paracrine response by these cells to maintain growth in the absence of exogenous growth stimuli. Alternatively one could interpret the data as actually being a down regulation of secretion of most of the cell lines in response to high serum.

HT1080 cells are an established human fibroblast cell line isolated from a fibrosarcoma [7]. HT1080 are not contact arrested and are considered tumorigenic in nude mice as a result of an activated N-ras phenotype [8]. Human mesothelial LP9/TERT-1 (LP9) cells are an hTERT-immortalized cell line phenotypically and functionally resembling normal human mesothelial cells [9]. H-Meso cells are an established cell line derived from a human malignant mesothelioma in 1987and has undergone a number of cytogenetic rearrangements [10]. GA and MI cell lines were isolated from two different individuals with malignant mesothelioma by the Vermont Cancer Center. From their origin, H-Meso, GA and MI cell lines would be expected to behave similarly, yet they have different phenotypes as well as different responses to serum. These differences can most easily be explained by the cell lines having undergone different multistage paths to cancer.

The differences between cell lines in regards to their response to serum are intriguing. Cells in culture are known to produce and secrete factors that stimulate modulate growth of themselves (autocrine) and neighboring cells (paracrine). In the presence of FBS, one would expect that all of the necessary growth factors are present to stimulate cells to grow. The increase in cancer markers under high serum conditions would seem to be a consequence of growth rather than a necessity for it.

Large numbers of samples make the use of automaton essential. The ELx50 Automated Microplate Strip Washer can be configured to wash either magnetic beads using magnets to immobilize the beads or polystyrene beads using a vacuum filtration manifold. Using for the wash steps is a tremendous help in terms of time and effort. The ELx50 carries out a wash process on an entire plate in less than 4 minutes while allowing for walk away operation. Besides being rapid and efficient, the washer provides consistent results for each wash step. The combination of multiplexing numerous assays in conjunction with the automation of wash steps allows for the generation of large amounts of sample data with minimal effort, reagent usage and time.

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Abbreviations

∞-fetoprotein (AFP) ;cytokeratine 19-fragment (CYFRA21-1); macrophage migration inhibitory factor (MIF); osteopontin (OPN);vascular endothelial growth factor (VEGF); Human choric gonadotropin (ß-HCG); Fibroblast Growth Factor 2 (FGF2); Transforming Growth Factor beta (TGF-ß); fetal bovine serum (FBS).