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Dissimilar cytokine patterns in different human liver and colon cancer cell lines

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ABSTRACT

An accurate and simultaneous estimate of cellular levels of a large cytokine number is very useful to obtain information about an organ dysfunction leading to cancer because through the understanding of the evolution of cytokine patterns we can recognize and predict the disease progression. Cancer cell lines are commonly used to study the cancer microenvironment, to analyze their chemosensitivity and carcinogenesis as well as to test *in vitro* the effect of molecules, such as drugs or anti-oxidants, on the inflammation status and its progression.

We noted that various cell lines commonly used as a model for studies on liver and colon cancer possess different patterns of cytokines. This aspect may generate data not comparable in laboratories using different cell lines; thus, to investigate the origin of these abnormalities we compared the cell lines HepG2 and Huh7, and HT-29 and HCT-116, for liver and colon cancer, respectively. In this context we have evaluated and compared the levels of cytokines, chemokines and growth factors in the supernatants of these cellular lines. Our aim was to identify what cytokines were significantly different correlating similarities and differences to the specific inflammation status of each cellular model of cancer.

1. Introduction

Inflammation is a physiologic process in response to acute tissue damage resulting from physical or ischemic injury, infection, exposure to toxins, chemical irritation, and other types of trauma [1,2]. When an inflammatory stimulus persists, the inflammation progresses and becomes chronic. In these cases various types of leukocytes, lymphocytes, and other inflammatory cells are activated and attracted to the inflamed site by a signaling network involving a great number of growth factors, cytokines, and chemokines [3]. All the cells recruited to the inflammatory site contribute to tissue breakdown [3]. The resolution of inflammation also requires a rapid programmed clearance of inflammatory cells by inducing apoptosis and conducting phagocytosis [4].

In the last years, it has been suggested a strong correlation between chronic inflammation and cancer [2,5,6]. In fact, chronic inflammation is involved in all three stages of tumor development: initiation, progression and metastasis [7]. Moreover, since many cancers arise from sites of infection, chronic irritation, and inflammation, it is now clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells and cytokines, is an indispensable participant in the neoplastic process altering not only the metabolic needs of the tissue, but also fostering DNA and protein damage, proliferation, survival, mutagenesis, migration and metastasis of malignant cells [8]. However, an important aspect of the tumor microenvironment is the cytokine mediated communication between tumor and cells. In fact, cytokines and chemokines show many activities that permit cell–cell communication locally at the tissue, with the outcome determined by the concentration of cytokines in the environment and the cell type [9]. Because the control of cytokine production is highly complex and multifactorial, the effects of cytokines are mediated through multiple regulatory networks. It is therefore informative to investigate the immunopathogenesis of a disease by analyzing patterns of cytokines and not cytokines considered individually [8]. At present, it is possible to effectively evaluate the patterns of cytokine levels using a broad-spectrum bead based multiplex immunoassay [10].

In our recent papers, we examined serum levels of cytokines, chemokines and growth factors in patients with type 2 diabetes and/or hepatitis C virus (HCV) and/or cirrhosis or with hepatocellular carcinoma (HCC) and HCV-related cirrhosis, using this multiplex immunoassay, to define a profile able to characterize these patients and to identify useful diagnostic and prognostic markers [11–14]. Moreover, we used this method also to evaluate the

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cytokine levels in patients with renal cell carcinoma (RCC) [15] and in patients with autoimmune Addison's disease [16].

Recently, we also evaluated the effects of lipoic and caffeic acids and of selenite on cytokinome profile in two human hepatoma cell lines using the multiplex immunoassay to study their putative anti-inflammatory effects [17,18]. Often we wondered how reliable are the results of similar cell lines but that originate from different individuals that have had the same type of cancer. This is because it is now evident the importance of the phenotype of the individual patient in determining his medical history and the evolution of the disease. Therefore, in this study we have evaluated the levels of cytokines, chemokines and growth factors in the supernatants of four different cancer cell lines, two of hepatoma (HepG2 and Huh7), and two of colon cancer (HT-29 and HCT-116), to identify what cytokines were significantly different in liver or colon cancer cells and to study similarities and differences of the inflammation status in cells used as model systems for each given cancer.

2. Methods

2.1. Cell culture

Cell cultures were kept in growth conditions identical or as similar as possible. Three human cancer cell lines, Huh7, HepG2, and HT-29 were kept in culture and expanded at 37 °C in a humidified atmosphere of 5% CO_2 in culture medium DMEM (Dulbecco's Modified Eagle's Medium, Lonza, Verviers, Belgium), supplemented with FBS (Invitrogen, Camarillo, CA, USA) at 10%, Penicillin/Streptomycin 100×(Euroclone, Devon, UK), Glutamax 100×(Invitrogen) and non-essential amino acids 100×(Invitrogen). The other human colon cancer cell line, HCT-116, was kept in culture and expanded in humidified atmosphere at 37 °C and 5% CO_2 incubator in culture medium RPMI1640 w/o L-Glutamine (Lonza, Verviers, Belgium), supplemented with FBS (Invitrogen, Camarillo, CA, USA) at 10%, Penicillin/Streptomycin 100×(Euroclone, Devon, UK), Glutamax 100×(Invitrogen) and non-essential amino acids 100×(Invitrogen). Phosphate buffer (PBS phosphate buffered saline Ca^{2+} and Mg^{2+} free) and trypsin (Ca^{2+} and Mg^{2+} free) were supplied by Euroclone.

2.2. Bio-plex assay

The multiplex biometric ELISA-based immunoassay, containing dyed microspheres conjugated with a monoclonal antibody specific for a target protein was used, according to the manufacturer's instructions (Bio-Plex Bio-Rad), to evaluate the levels expressed as fluorescence intensity of different cytokines by Human Cytokine 27-Plex Panel in supernatants of Huh7, HepG2, HT-29 and HCT-116 cells. In particular, the following cytokines were evaluated: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin (CCL11), basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), PDGF-ββ, RANTES (CCL5), TNF-α and VEGF. Each experiment was performed in duplicate as previously described [11–18]. Protein levels were determined using a Bio-Plex array reader (Luminex, Austin, TX, USA) that quantitates multiplex immunoassays in a 96-well format with very small fluid volumes.

2.3. Bioinformatics analysis

The cytokines fluorescence intensities evaluated in the cellular supernatants were compared by *T*-test by the statistical program Prism 4 (GraphPad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered to be statistically significant. Moreover, the levels of these molecules were analyzed with the Cluster 3.0 program using a clustering algorithm applied by similarity metrics based on a Pearson correlation that builds a hierarchical structure among objects (molecules and cells) and shows a correlation [19]. The TreeView program was used for visualizing and browsing the clustered data.

3. Results and discussion

Many studies have evidenced the link between chronic inflammation and cancer [20,21] and the important role played from cytokines, chemokines and growth factors in cancer progression and metastasis [22]. The simultaneous quantitative determination of a large panel of cytokines, able to report the correct ratios and dynamics between highly and poorly represented molecules, is emerging as an accurate, simple, specific, noninvasive, reproducible and less expensive method [11–18]. On the other hand the cancer cell lines are commonly used as model systems to evaluate the biological effects of molecules like drugs or antioxidants as well as to understand if these molecules have anti-inflammatory effects. However, our recent studies on the evaluation of the effects of selenite, and lipoic and caffeic acids on two hepatoma cell lines (HepG2 and Huh7) evidenced that these molecules induced similar but not identical effects because they presented different inflammatory status [17,18]. Hence, in our opinion it is imperative to analyze differences and similarities in cancer cell lines used as model of a given cancer. This is an important point to obtain homogenous experimental results and evaluation. Hence, using a multiplex biometric ELISA-based immunoassay we evaluated the levels of different cytokines by Human Cytokine 27-Plex Panel in supernatants of two cell lines of liver cancer, HepG2 and Huh7, and two colon cancer, HT-29 and HCT-116. We have chosen to focus the attention on these two cancers because they are among the first five mortality causes in Italy. Moreover, a high occurrence of liver metastasis is reported during the development of colon cancer [23].

3.1. Cytokine evaluations in HepG2 and Huh7

In Fig. 1 and in Table 1 we report the cytokine levels evaluated as fluorescence intensities (FI) in the two liver cancer cell lines, i.e., HepG2 and Huh7. In details, radar graphs clearly evidenced that many cytokines (i.e. IL-8, IL-12, IP-10, MCP-1, MIP-1β and VEGF) had higher levels in Huh7 in respect to HepG2.

To assess how significant are these different cytokine levels in the two cancer cell lines, we compared the cytokine levels also by *T*-test. This analysis showed that the following cytokines have significantly higher levels in Huh7: (i) IL-9 and IL-13 with p-value < 0.05, (ii) IL-6, IL-10, IL-12, G-CSF, MIP-1α and RANTES with p-value < 0.01 and (iii) IL-8, MCP-1, MIP-1β, IP-10 and VEGF with p-value < 0.0001 (see Table 1S).

The levels of these cytokines are strictly correlated between them because IL-12 has a strong anti-angiogenic activity and stimulates the production of IP-10 [24] whereas G-CSF induces activation, differentiation, and growth of granulocyte, as well as it is involved in inflammatory processes via macrophage activation and proliferation [11]. Furthermore, IL-8 is a pro-inflammatory chemokine having a strong pro-angiogenic activity in HCC patients and its expression in HepG2 cells has been correlated with invasiveness of tumor metastasis because it was found increased in the later stages of HCC [17]. On the other hand, IL-6 acts as pro-inflammatory as well as anti-inflammatory cytokine and stimulates the secretion of VEGF which is an established potent angiogenetic factor with pro-inflammatory properties [25]. As far as IL-10 is concerned, it shows a direct effect on apoptosis by blocking the activation of NF-kB, and its increase can lead to programmed cell death of tumor cells [26,27]. Whereas the four chemokines MCP-1, MIP-1α, MIP-1β and RANTES, act in a correlated way and play a role in HCC progression and in cell