Original Article

Multiplex Analysis of Cytokines Involved in Tumour Growth and Spontaneous Regression in a Rat Sarcoma Model

(cytokines / sarcoma cells / spontaneous regression / antibody array)

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Abstract. The aim of our study was to examine *in vivo* and *in vitro* cytokines produced by Lewis rat-derived R5-28 sarcoma cells. These cells produce rapidly growing tumours in approximately two weeks after subcutaneous inoculation. However, spontaneous tumour regression was noted in about 40% of animals. For an explanation of this phenomenon, we evaluated the profile of 19 cytokines during tumour growth and spontaneous regression by the use of „antibody array". To detect cytokines directly originated by the sarcoma, the R5-28 cells were cultivated *in vitro* and then both the supernatants and the cell lysates were analysed. Our experiments showed three cytokines (MCP-1, TIMP-1 and VEGF) to be produced by R5-28 cells *in vitro*. Moreover, *in vivo*, another three cytokines (TNF-α, β-NGF and LIX) were detected both in blood sera and tumour lysates, probably produced by immune and stromal cells during tumour growth. Changes in their expression after spontaneous regression are discussed.

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Abbreviations: CINC-2, CINC-3 – cytokine-induced neutrophil chemoattractant 2 and 3, CNTF – ciliary neurotrophic factor, GM-CSF – granulocyte/macrophage colony-stimulating factor, IFN-γ – interferon γ, IL-1α – interleukin 1α, IL-1β – interleukin 1β, IL-4 – interleukin 4, IL-6 – interleukin 6, IL-10 – interleukin 10, LIX – LPS-induced CXC chemokine, MCP-1 – monocyte chemoattractant protein 1, MIP-3α – macrophage inflammatory protein 3α, β-NGF – β nerve growth factor, TIMP-1 – tissue inhibitor of metalloproteinase 1, TNF-α – tumour necrosis factor α, VEGF – vascular endothelial growth factor.

Introduction

Tumour cells commonly participate in shaping their own microenvironment by producing various cytokines and chemokines. These molecules can influence tumour growth by three important mechanisms: regulation of tumour-associated angiogenesis, modulation of host anti-tumour responses, and direct stimulation of tumour cell proliferation (Frederick et al., 2001). They can also sustain the direct migration of leucocytes, their adhesion to endothelia and subsequent trans-endothelial migration inside tumours. The stimulation of angiogenesis (e.g. by vascular endothelial growth factor (VEGF)) and tumour growth can also be controlled by chemokines and cytokines either of cancer cell origin or produced by tumour-recruited macrophages and other immune cells (e.g. tumour-infiltrating leucocytes (TIL)) (Vicari and Caux 2002; Shurin et al., 2006).

The R5-28 sarcoma cells were established in our laboratory from a spontaneously arising Lewis rat cancer. These cells showed high proliferating capacity, vimentin positivity and cytokeratin 18 negativity (Morávková et al., 2005). When inoculated in syngenic animals, these cells can produce tumours either rapidly developing or going to regress. In this preliminary study we wanted to screen a panel of cytokines involved in tumour evolution to compare the two conditions.

Material and Methods

Three months old male Lewis rats were obtained as inbred and pathogen-free animals from the Institute of Physiology AS CR, v. v. i. (Prague, Czech Republic).

They were housed in the animal facility of our Institute, under controlled light-dark cycling. Feeding by standard-pellets diet and water intake were unrestricted. After one month of adaptation in the new environment, the rats were subcutaneously inoculated in the back with 5×10^5 of R5-28 cells (2nd passage, cultivated in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum), suspended in 0.1 ml phosphate-buffered saline. The animals were then divided according to the tumour growth into two groups: 1) with tumour progression (6 animals), and 2) with tumour regression (5 animals). Three healthy animals served as a control. The sarcoma progression or regression was evaluated by measuring the tumour dimensions every three days. At the fourth week after inoculation of the sarcoma cells, the progression or regression process was clearly defined. At that time, blood samples were collected from the tail veins. The blood, without any additive, was left to clot at room temperature for 1 h, centrifuged at 1500 × *g* for 10 min at 4 °C, and the obtained sera were stored at –80°C. Cytokines were studied both in the blood serum and in tumour samples. To distinguish the cytokines produced by either the tumour or the microenvironmental components, we also analysed medium and cell lysates from *in vitro* cultivated R5-28 cells. Samples from growing and spontaneously regressing tumours were excised under total anaesthesia, immediately frozen in liquid nitrogen, and frozen sections (8 μm) were prepared in a Leica CM 1850 (Leica Instruments Gmb, Nussloch, Germany) cryocut for protein extraction. A mean of 100 sections from each sample were collected and treated with 1 ml of lysis buffer (RayBio Rat Antibody Array I, 1.1 Kit, RayBiotech Inc., Norcross, GE) on ice for 30 min, and then centrifuged at 12 000 × *g* for 20 min at 4 °C. Protein concentration in supernatants was determined by a BCA Protein kit (Pierce Inc, Rockford, IL). For the *in vitro* study, the R5-28 cells were cultivated for three days and the cultivation medium was separated and stored at –80°C. Cell pellets were lysed with the lysis buffer. Protein concentration was determined as indicated above, adjusted to concentration 500 μg/ml of total protein and stored at –80°C. A commercially available antibody array (RayBio Rat Antibody Array I, 1.1 RayBiotech Inc.) for multiplex analysis of 19 rat cytokines (CINC-2, CINC-3, CNTF, Fractalkine, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, LIX, Leptin, MCP-1, MIP-3α, β-NGF, TIMP-1, TNF-α and VEGF) was used for screening their expression. Signals were measured with a chemiluminescence-imaging detector (LAS 1000, FujiFilm, Tokyo, Japan) and also developed on CL-Xposure Film (Pierce Inc). Analysis of spots was performed with ImageJ software (version 1.36b, National Institutes of Health, Rockville Pike Bethesda, MD). Intensity of each individual spot was normalized by comparison to 100% intensity of positive controls. The results were calculated by MS Excel (Microsoft, Washington, DC) and expressed as a mean ± SD. Differences were evaluated by the Student's *t*-test and the value of $P < 0.05$ was considered statistically significant.

Results and Discussion

We found that the R5-28 cells secrete monocyte chemoattractant protein-1 (MCP-1) *in vivo* and *in vitro*. As reported in the literature, the biological effect of tumour-derived MCP-1 is double-faced. Low secretion with modest monocyte infiltration sustained tumour growth by increased angiogenesis. High secretion was associated with massive monocyte/macrophage infiltration inside the cancer mass leading to its destruction. Tumour cells transfected to express various levels of MCP-1, once inoculated in mice, demonstrated direct correlation between the MCP-1 production and intratumoral macrophage infiltration (Nesbit et al, 2001). Moreover, MCP-1, by attracting activated T cells, natural killer (NK) cells, basophils and monocytes, modulated Th1 immune response (Omata et al, 2002), possibly playing an important role in the spontaneous regression process observed in our model.

VEGF and tissue inhibitor of metalloproteinase 1 (TIMP-1) were found to be expressed by the R5-28 cells both *in vivo* and *in vitro* (Fig. 1). VEGF is the most important molecule involved in angiogenesis, a critical event for the growth and progression of neoplasms. Tumours are dependent on neovascularization to sustain their expansion, and can themselves produce pro-angiogenic factors (Narazaki and Tosato, 2006). VEGF is also a key factor produced by solid tumours to inhibit immune recognition (Ohm and Carbone, 2001). Generally,

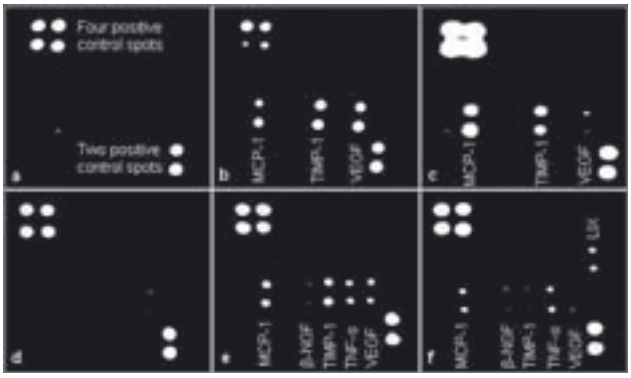

Fig. 1. Antibody array detection of cytokines in the R5-28 rat sarcoma model. a, b, c: *in vitro* experiments with the R5-28 cells, d, e, f: *in vivo* experiments, a. cultivation medium (negative control), b. medium collected after 3-day cultivation of the R5-28 cells, c. intracellular cytokine profile of R5-28 cells (cell lysates), d. serum from a healthy animal, e. lysate from a growing tumour (500 μg/ml of total protein concentration), f. lysate from a tumour with spontaneous regression. Each membrane contains positive and negative controls. Representative data are shown.

overexpression of TIMP-1 has been associated with decreased tumorigenicity and metastatic capability of various cancer cell that present upregulation of matrix metalloproteinases (Tsuchiya et al, 1993; Baker et al, 1994; Khokha 1994; Jiang et al, 2002; Ikenaka et al, 2003). However, TIMP-1 also showed paradoxical effects on tumour progression and growth in some models. It was e.g. capable to upregulate VEGF, leading to increased growth of rat mammary carcinoma cells in nude mice (Yoshiji et al., 1998). In clinical works, higher levels of TIMP-1 in the serum of cancer patients were also described as a negative prognostic factor (Kallakury et al., 2001).

In our model we found that the levels of MCP-1, VEGF and TIMP-1 were significantly reduced ($P < 0.05$) in rats with spontaneous regression in comparison to animals with growing tumours (Fig. 2). Overexpression of TIMP-1 may explain why the R5-28 cells do not show any metastatic potential. Other three cytokines – tumour necrosis factor α (TNF-α), β nerve growth factor (β-NGF) and LPS-induced CXC chemokine (LIX) – were detected *in vivo* in serum samples during tumour growth. However, their levels were not significantly different from those found in rats with spontaneous regression, according to semi-quantitative densitometry analysis (Fig. 2). LIX showed significant overexpression in lysate samples from tumours with spontaneous regression (Fig. 1). The role of these cytokines in tumour progression varies and their ratio might be responsible for unique tumour properties. TNF-α, mainly produced by activated macrophages, T lymphocytes and NK cells, has potent tumour –suppressing activity and even induces haemorrhagic necrosis in some types of tumours (Van Horssen et al., 2006). LIX may enhance proliferation and invasion of squamous cell carcinomas (Miyazaki et al., 2006). LIX also showed potent chemoattractant activity for neutrophils *in vitro* and *in vivo* (Wuyts et al., 1996; Chandrasekar et al., 2001). β-NGF belongs to the family of neurotrophins with ability to modulate several human malignancies (Pahlman and Hoehner, 1996). The role of β-NGF is rather ambiguous since it is involved in stimulation of clonal growth in human lung cancer cells *in vitro*, but also can slow down or arrest the neoplastic progression (Pahlman and Hoehner, 1996; Ricci et al., 2001).

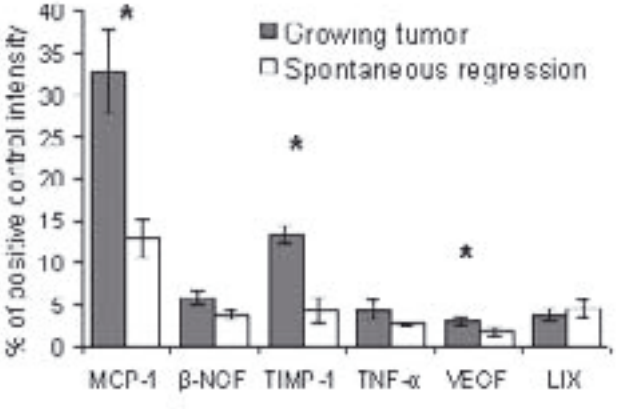

Fig. 2. Changes in the expression levels of six cytokines in blood serum – rats with a growing tumour versus rats with spontaneous regression. (*$P < 0.05$, Student's *t*-test). Densitometry analysis performed with ImageJ software.

Cancer cells are able to produce cytokines to conditionate the microenvironment in which they develop. These cytokines are important for modulation of angiogenesis and immune cell chemoattraction. Spontaneous regression, the extremely rare event in several types of tumours, is a common phenomenon in our rat model. Generally, it is accepted that the main anti-tumour response is of Th1 type and tumour-related immune changes may associate with inadequate synthesis of Th1 cytokines (IL-2, IL-12, IFN-γ) (Lauerová et al., 2002). The membrane array (designed by the RayBiotech) that we used in this experiment enables investigation of a limited spectrum of Th1 and Th2 cytokines. However, no detectable Th2 (IL-4, IL-6, IL-10, data not shown) or Th1 cytokine expression was present in both growing and regressing tumours. Therefore, further studies to better define the functional and biological characteristics of R5-28 cells (e.g. analysis of relevant cytokines with ELISA tests and phenotypic characterization of both cytokine-producing cells and tumour-infiltrating lymphocytes by flow cytometry) are under way to assist a more complete explanation of the regression phenomenon.

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