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The involvement of granulocytes in spontaneous regression of Walker 256 carcinoma

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Abstract

We described before that oxidative burst of granulocytes is cytotoxic for melanoma B16F10 and for Walker 256 carcinoma (W256). Therefore, we assumed that granulocytes could also be important mechanism of the host defence against tumour. In current study we report massive granulocyte infiltration at the site of W256 transplanted in the hind limb of Sprague–Dawley associated with spontaneous tumour regression observed for 22/25 rats (87%). Peripheral blood granulocytes of these animals were highly cytotoxic for W256 cells cultured in vitro. After the tumour disappearance the inflammatory oxidative burst of the granulocytes ended. Distraction of granulocytes from the tumour by s.c. Sephadex injection decreased the incidence of the W256 regression to only 7/25 animals (30%). These results suggest that innate immunity based on immune competent granulocytes may be the cause of well known phenomenon of spontaneous regression of W256 carcinoma.

Keywords: Oxidative burst; Granulocytes; Walker 256 carcinoma; Anti-cancer effects; Spontaneous tumour regression; Sephadex

1. Introduction

While it is well known that polymorphonuclear leukocytes play an essential role in host defence against microorganisms [1], mechanisms by which granulocytes may be involved in immune reactions against cancer are not understood. It was recently reported that spontaneous regression of or complete resistance to cancer cells is mediated by rapid infiltration of leukocytes, mostly of innate immunity [2]. Several researchers have reported cytotoxicity of granulocytes against tumour cells *in vitro* [3–6] and *in vivo* [7]. The activation process of granulocytes is accompanied by the intense production of reactive oxygen species (ROS) [1,8] and an extended release of destructive hydrolytic enzymes [9,10]. It was demonstrated that the activated granulocytes cause unspecific lysis of tumour cells mediated by ROS [11–13]; hence ROS have been identified as effector molecules in the mechanisms of oxygen-dependent

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killing of cancer cells by granulocytes [13,14]. However, in spite of many data showing that granulocytes can participate in the destruction of malignant cells, little is known about the function of granulocytes in tumour-bearing hosts.

In the previous study [15], we analysed the oxidative burst and anti-tumour activities of murine neutrophil granulocytes when challenged with B16–F10 melanoma. Mice were intramuscularly injected with melanoma B16–F10 and/or subcutaneously with granulocyte attractant substance Sephadex G-200. Intensive oxidative burst was determined by chemiluminescence of neutrophils obtained from the papula developed at the site of Sephadex injection. These granulocytes were cytotoxic for the B16-F10 cells *in vitro*. However, survival of Sephadex injected tumour-bearing mice was lower than of control animals bearing B16–F10, while their tumours were less necrotic and grew faster. We therefore concluded that injection of Sephadex distracted the neutrophils from the tumour cells allowing faster progression of the tumour, indicating that neutrophils may have an important role in the host defence against malignant cells in the early stage of tumour development.

To test further this possibility we used in this study the W256 carcinoma model grown on Sprague–Dawley rats. Namely, in the earlier study using W236 carcinoma grown on Wistar rats we found that functional granulocytes could be obtained from the Sephadex papula of the normal, healthy rats that act against the W256 tumour cells *in vitro* [16]. Moreover, the presence of the W256 tumour cells *in vitro* induced oxidative burst of the neutrophils as much as the phorbol ester, which is the most potent inducer of the oxidative burst. However, the granulocytes from the Sephadex papula of the tumour-bearing animals were less reactive *in vitro* than were granulocytes from the Sephadex papula of the normal healthy animals, indicating that the most potent granulocytes were already engaged in reaction against the W256 *in vivo*. However, we did not have histological evidence for this assumption.

Therefore, aiming to monitor the role of granulocytes in relation to tumour progression or regression, in this study we performed histological analyses of W256 at different time points after tumour transplantation in the hind limb. On the other hand, instead of Wistar rats we transplanted W256 in the Sprague–Dawley rats. Namely, it is well known that W256 exerts different growth patterns depending on the strain of the host rats used and that even spontaneous regression of the W256 could be observed [17,18]. However, so far the phenomenon of spontaneous regression of W256 was not explained. We assumed that early anti-cancer reaction of the granulocytes could be involved in spontaneous regression of W256, which would prove the potential of granulocytes to act as efficient anti-cancer immune competent cells against W256. To confirm that beside the histology carried out in consecutive days during W256 regression we used the group of animals treated with s.c. Sephadex injection to distract their granulocytes from the tumour into the remote Sephadex papula.

Finally, to verify if circulating granulocytes could be responsible for the regression of W256, we compared the oxidative burst and the anti-tumour activity *in vitro* of granulocytes from the blood of the animals with spontaneously regressing tumours with the activity of the blood granulocytes obtained form the normal healthy animals without tumour.

2. Materials and methods

2.1. Animals and treatment

All experiments were performed on male, 5-months-old Sprague–Dawley rats with water and food given *ad libitum*. Animals were intramuscularly injected in the right hind limb with 250 μL of RPMI medium containing 10^7 W256 tumour cells only (W group) harvested from Wistar W256 bearing donors or additionally with Sephadex (WS group) (Sephadex G-200, Pharmacia Fine Chemicals, Sweden). Sephadex was used as described elsewhere [15]. There were 25 animals per group and their survival was monitored daily. All the experiments were performed in accordance with the ILAR Guide for the Care and Use of Laboratory Animals, Council Directive (86/609/EEC) and Croatian Animal Welfare Law (NN 19/99).

2.2. Functional activity of circulating granulocytes

Distribution of granulocytes in the peripheral blood and functional activity of circulating phagocytes (measured by chemiluminescence assay) were determined 4, 7 and 10 days after tumour transplantation as described elsewhere [16]. Leukocytes in the blood were counted in the Bürker chamber using Türk's solution (Kemika, Croatia). In order to determine the respiratory burst of circulating granulocytes (ROS production), 10 μL of EDTA-containing blood samples, isolated from the tail vein, were mixed with 10 μL of PMA (1 μg/mL, phorbol-12-myristate-13-acetate, Sigma–Aldrich, USA) and diluted in 600 μL of chemiluminescence mixture [19]. Chemilumines-