Immunohistochemical Evidence of the Involvement of Natural Killer (CD161⁺) Cells in Spontaneous Regression of Lewis Rat Sarcoma

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Abstract. *Background/Aim: Spontaneous regression (SR) of tumours is a rare phenomenon not yet fully understood. The aim of this study was to investigate immune cells infiltrating progressive and SR tumours in a Lewis rat sarcoma model. Materials and Methods: Rats were subcutaneously inoculated with rat sarcoma R5-28 (clone C4) cells. Developing tumours were obtained on day 42 and cryosections were immunohistochemically processed for detection of immune cells. Results: A high density of granulocytes was found in the necrotic areas of both progressive and SR tumours. CD4⁺ cells and CD8⁺ cells were rare and sparsely dispersed in the tumour tissue without clear difference between the two types of tumours. On the contrary, CD161⁺ cells were abundant and evenly distributed in SR tumours, but these cells were very rare in progressive tumours. Conclusion: Based on the differences in number and distribution of the immune cell subpopulations, we believe that natural killer (CD161⁺) cells play a major role in the destruction of cancer cells during SR of tumours in this Lewis rat model.*

Spontaneous regression (SR) is a partial or complete disappearance of a tumour in the absence of any treatment. It is a rare phenomenon which can be found in primary as well as metastatic tumours. Its frequency is dependent on the

histological type of tumour. The immune system and its mediators (cytokines/chemokines) participate in this process (1, 2). Granulocytes take part in SR of some tumour types because of their ability to recognize tumour cells, inhibit their proliferation and remove tumour tissue using phagocytosis (3). On the contrary, granulocytes can also activate tumour progression by supporting tumour angiogenesis (4). They are also able to defend tumour cells from immune-system attack by their suppressive effect against T-lymphocytes (5). This dual behaviour can be explained by polarization of tumour-infiltrating granulocytes under the tumour milieu into two phenotypes. A tumour-promoting phenotype is supported by transforming growth factor (TGF) β , whereas a tumour-suppressing phenotype appears in the absence of TGF β (6, 7) or under the influence of type I interferons (8).

Various subsets of lymphocytes producing pro-tumorigenic or anti-tumorigenic cytokines can also promote tumour progression or suppress tumour development. Natural killer (NK) cells and CD8⁺ cytotoxic T-lymphocytes (CTL) are the main representatives of innate and adaptive anticancer immunity, respectively (9). Simply put, CD4⁺ T-helper (Th) cells differentiate into four basic subpopulations with antitumour effects (Th1 cells) or with tumour-supporting (Th2 cells, Th17 cells) or immunosuppressive activity [Treg (T-regulatory) cells] (10, 11). Populations of lymphocytes in tumours show considerable heterogeneity depending on cancer type, its immunogenicity and cytokine milieu. The density, type and pattern of distribution of tumour-infiltrating lymphocytes (TILs) and their respective ratios have been revealed as valuable prognostic and therapeutic biomarkers in patients with ovarian (12, 13), colorectal (14), and breast (15) cancer, and melanoma (16, 17).

A thorough understanding of the mechanisms of SR could help improve approaches to cancer therapy. However, the process of SR cannot be studied in detail in humans due to

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ethical standards. For this reason, there are various animal tumour models serving well in this respect. We developed a Lewis rat sarcoma model at the Institute of Animal Physiology and Genetics in Libeňov that demonstrates progression and SR of subcutaneous tumours arising after inoculation of cancer cells. The R5-28 cell line was established from a spontaneous sarcoma found in a female Lewis rat and three distinct clones (C4, C7, and D6) were derived from the original cell line. The R5-28 cell line and all three clones were characterized *in vitro* and *in vivo* in terms of cell and tumour growth, cytokine and protein expression and changes of haematological and immunological parameters (18-22). More than half of the animals inoculated with clone C4 cells showed SR of subcutaneous tumours, whereas only progressive tumours were found after inoculation with cells of clones C7 and D6 (20). This study focused on analyses of immune cells infiltrating both progressive and SR tumours. For this reason, we chose clone C4 for inoculation of the Lewis rats.

Materials and Methods

Cells. Clone C4 (the 4th passage) R5-28 rat sarcoma cells were cultivated in high-glucose Dulbecco's modified Eagle's medium (Sigma-Aldrich, Prague, Czech Republic) supplemented with 10% heat inactivated foetal bovine serum (Lonza, Basel, Switzerland), 10 IU penicillin/5 µg streptomycin per 1 ml, 10 µM HEPES, 1 mM L-glutamine and 1 mM non-essential amino acids (all from Sigma-Aldrich) in 5% CO₂ at 37°C. Growing cells (reaching almost 100% confluency) were washed with phosphate-buffered saline (PBS), detached using 0.2% trypsin-EDTA (5 min/37°C) and centrifuged (after addition of the same volume of culture medium) at 112 × *g* (5 min). The cell suspension was then washed twice in PBS, diluted to the desired cell density with PBS and subcutaneously injected into the dorsal side of experimental animals.

Animals. Inbred female Lewis rats, 70 days old (Velaz s.r.o., Prague, Czech Republic), were used in this experiment. They were housed in the animal facility at the Institute of Animal Physiology and Genetics in Libeňov under a controlled light-dark cycle. Rats were fed with a standard pellet diet (ST-1 feed mixture; Velaz s.r.o.) and water *ad libitum*. The clone C4 R5-28 rat sarcoma cells were applied subcutaneously at dose of 5×10⁵ in 0.2 ml PBS to 21 experimental rats. Sarcoma progression and regression were evaluated on the basis of tumour size (measured twice a week). Four healthy animals injected subcutaneously only with 0.2 ml PBS (without any cells) served as a control group.

The first subcutaneous tumours were macroscopically observed on day 21. Tumour samples for further analyses from six rats were collected on day 42 when progressing tumours had reached a very large size (around 40×25 mm), rats bearing them showed cachexia and had to be euthanized for ethical reasons. Four animals with SR tumours were sacrificed on day 49 in order to collect tumour samples for further analyses, when SR was visual and before the tumour tissue was completely absorbed. The remaining four animals with SR continued to be observed for complete tumour disappearance and were sacrificed on day 66 (*i.e.* the end of study).

More results are published in our previous article (21). Halothane inhalation followed by decapitation was used for euthanasia. This experiment was performed in accordance with the Project of Experiments number 099/2011 approved by the Central Animal Science Committee of the Czech Academy of Sciences, following the rules of the European Convention for the Care and Use of Laboratory Animals.

Histology and immunohistochemistry. All sarcomas were excised after euthanasia. Tumour samples (about 8×8×10 mm) were immediately taken, frozen in liquid nitrogen and stored at -80°C for further histological and immunohistochemical analysis. Tissue cryosections (7 µm) were prepared using a Leica CM 1850 cryostat (Leica Instruments GmbH, Nussloch, Germany). For histological evaluation of tumours, the cryosections were processed for haematoxylin-eosin staining by routine procedures. Briefly, the sections were washed with distilled water, fixed with ethanol (20 min), washed three times with distilled water (5 min each) and treated with Weigert's haematoxylin (20 min) for nuclear staining. After washing with running tap water (20 min) followed by distilled water (3×5 min), the cytoplasm was counterstained with 1% eosin alcoholic solution (2 min), then the stained sections were washed with distilled water (3×5 min) and embedded in glycerine jelly.

Indirect immunofluorescence was applied for detection of various types of immune cells infiltrating the tumour tissue. Cryosections were fixed in acetone (-20°C, 15 min), washed in PBS (3×5 min) and blocked with 10% rat serum (room temperature, 60 min). Sections were then incubated in the following solutions with primary monoclonal antibodies diluted 1:100 with 10% goat serum (refrigerator, overnight): mouse anti-rat CD4 (Abnova, Taipei, Taiwan), mouse anti-rat CD8α (Abnova, Taipei, Taiwan), mouse anti-rat CD161 (LifeSpan BioSciences, Seattle, WA, USA) and mouse anti-rat granulocyte/fluorescein isothiocyanate (FITC) (eBioscience, San Diego, CA, San Diego, USA). After careful washing with PBS (3×5 min), secondary antibodies Alexa Fluor 555-conjugated goat anti-mouse IgG2a and Alexa Fluor 488-conjugated goat anti-mouse IgG1 (both Invitrogen, Grand Island, NY, USA) diluted 1:1500 with 10% goat serum were applied (room temperature, 60 min) to detect the bound primary antibodies. Sections were again washed with PBS (3×5 min) and nuclei were counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich) at room temperature for 5 min. Finally, the stained sections were briefly washed with distilled water and embedded in Mowiol (prepared according to technical datasheet No. 777; Polysciences, Inc., Warrington, PA, USA) with N-propyl gallate (at final concentration of 5 mg/ml; Sigma-Aldrich). Mouse IgG1 Isotype control/FITC and mouse IgG2a Isotype control/FITC (both Invitrogen) were diluted 1:100 with 10% goat serum and utilized in control sections. A BX51 fluorescence microscope (Olympus Czech Group, s.r.o., Prague, Czech Republic) with Infinity 2 CCD Monochrome Microscopy Camera (Lumenera Corp., Ottawa, ON, Canada) and QuickPhoto Micro 2.3 software (Promicra s.r.o., Prague, Czech Republic) in pseudo-colour mode were used for qualitative evaluation of the immunohistochemically stained sections.

Results

Progressive subcutaneous tumours appeared in six rats and SR tumours in eight rats, *i.e.* in 28.6% and 38.1% of all animals, respectively. Seven rats (33.3%) did not develop

Figure 1. Comparison of rat with tumour and progression (A-C), and spontaneous regression (D-F). Overall aspect of animals with subcutaneous tumours (A, D), subcutaneous tumours after excision (B, E) and their cross-sections (C, F) clearly document the large size of the progressive tumour and much smaller size of the tumour with spontaneous regression.

any tumour. Both progressive and SR tumours were detectable macroscopically at the same time (on day 21) and they grew similarly until day 30. From that time, the progressive tumours started to develop more rapidly so that on day 42 they were more than twice as large as SR tumours and animals bearing them had to be sacrificed for ethical reasons. SR tumours had shrunk by about one-third by day 49 and were not detectable on day 66. The detailed data of tumour size have already been given in our previous article (21). Rats of the control group (with subcutaneous injection of 0.2 ml PBS) exhibited only a very small bump at the site of PBS application, which disappeared within a few days (Figure 1).

All progressive, as well as SR tumours were freely movable and completely covered with connective tissue. Both types of tumour showed great necrotic areas. In addition, cyst formation was occasionally observed in progressive tumours. Necrotic tissue was clearly demonstrated histologically. It was separate from the native tissue, showing marked destruction of cancer cells and leukocyte infiltration (Figure 2A). Moreover, pronounced vascularization and rebuilding of destroyed tumour tissue into fibrous tissue was found in SR tumours (Figure 2B).

Immunohistochemical analysis revealed a very high number of CD161⁺ cells that were evenly dispersed throughout the whole SR tumour tissue sections (Figure 2D). On the contrary, these cells were very rare and uniformly sparsely distributed in progressive tumours (Figure 2C). No clear differences between progressive and SR sarcomas were found in the level of infiltration and distribution of CD4⁺ cells (Figure 2E and F), CD8⁺ cells (not shown) and granulocytes (Figure 2G and H). CD4⁺ cells and CD8⁺ cells were rare and irregularly dispersed in the tissue.

Granulocytes were unevenly distributed in both progressive tumour without (Figure 2E) and with necrosis (Figure 2H), and SR (not shown) tumours. Considerable accumulation of granulocytes was ascertained in necrotic areas. On the contrary, their density in non-necrotic tissue was very low or they were not observed at all. Control sections with isotype controls did not show any positively stained cells.

Discussion

Clone C4 R5-28 rat sarcoma cells were inoculated subcutaneously into inbred Lewis rats and we monitored growth of developing tumours and analysed them immunohistochemically with the aim of characterizing tumour-infiltrating immune cells. The whole group of experimental animals were split up into three approximately equal groups according to their tumour development, namely rats with progressive tumours, rats with SR tumours, and rats without any tumour growth. This finding shows that even the inbred strain of rats is not homogeneous concerning reactivity to sarcoma cells and about one-third of Lewis rats were able to suppress cancer cells totally. This suggests differences among individual rats in immune cells participation in anticancer response or in expression of cytokines activating/suppressing them. The exact mechanisms triggering this process are not clear.

Considerable infiltration of SR tumours by CD161⁺ cells was noted immunohistochemically in this experiment. On the contrary, we found only negligible amount of CD161⁺ cells in progressive tumours. This difference in distribution of CD161⁺ cells in progressive and SR tumours suggests recruitment of these immune cells into SR tumours. CD161, C-type-lectin-like membrane glycoprotein receptor (also known as NKR-P1), is

Figure 2. Haematoxylin-eosin staining of tumour with progression (A) and spontaneous regression (SR) (B). Massive necrosis with leukocyte infiltration is visible in progressive tissue (*). In SR tumours, similar necrotic areas were also found (not shown). In addition, vascularization (arrows) and rebuilding of destroyed tumour tissue into fibrous tissue is evident (B). Magnification $\times 200$. Immunohistochemical detection of CD161⁺ cells (C, D), CD4⁺ (E, F) and granulocytes (G, H) by indirect immunofluorescence in progressive (C, E, G, H) and SR (D, F) tumour tissue (both without necrotic areas). Distribution of granulocytes in progressive tumour tissue without (G) and with necrosis (H) is shown. Cells stained positively by the antibody are green, cell nuclei are counterstained with 4',6-diamidino-2-phenylindole (blue). Magnification $\times 400$.

primarily associated with NK cells (23). Moreover, CD161 has been also identified on subsets of human CD4⁺ and CD8⁺ T-cells. These CD161⁺ T-cells represented the minority of blood T-cells in healthy humans (24, 25). In peripheral blood and tumour tissue of patients with cancer, CD8⁺CD161⁺ cells were found in low numbers, but CD4⁺CD161⁺ cells were significantly increased (26). In the rat, CD161 has been revealed as an activating cell-surface receptor for NK cells (27). In addition, a low level of CD161 expression was observed on a small subset of rat T-cells (28). Thus, it is clear

that the CD161 is not a fully specific marker of NK cells. Because immunohistochemically we found only a very low number of CD4⁺ and CD8⁺ in SR tumours, we deduce that the high CD161⁺ cell infiltration represents NK cells.

Unlike CD161⁺ cells, rat progressive and SR tumours did not differ in their infiltration by granulocytes. In both tumour types, we observed necrotic areas with a high density of granulocytes. Various cytokines and chemokines attract this type of natural phagocyte to tumour tissue (29). Previous study of the clone C4 R5-28 rat sarcoma cells *in vitro* revealed that these cancer cells release into cultivation medium cytokine-induced neutrophil chemoattractant 2 and monocyte chemoattractant protein 1 (20), two cytokines with chemoattractant activity for granulocytes (30, 31). Thus, it seems that the clone C4 R5-28 rat sarcoma cells alone could be directly responsible for migration of granulocytes into subcutaneous tumours. As already mentioned (6, 7), tumour-associated granulocytes can be polarized into cells with tumour-suppressing or tumour-promoting phenotype with completely different functional properties. The mouse monoclonal antibody which we applied for the detection of granulocytes (clone HIS48; eBioscience) cannot distinguish between these two phenotypes because it reacts with a molecule expressed on all rat granulocytes. Thus, we cannot say whether the granulocytes in progressive and SR tumours represent different phenotypes (or different mixtures of both phenotypes) nor to what extent they might participate in growth or SR of subcutaneous tumours in the Lewis rat sarcoma model. To resolve this problem, it would be necessary to determine the cytokine profile of granulocytes in both types of tumours.

There are also other players among immune cells known to influence tumour progression or suppression. CD4⁺ T-cells differentiate depending on the cytokine milieu into Th1, Th2, Th17 and Treg subpopulations with distinct effector functions and cytokine profiles. These subpopulations influence each other and also have an impact on other types of immune cells (10, 11). Their clinical significance was already noted *e.g.* in patients with oral cancer (32) and colorectal cancer (33). It was found that the Th1/Th2 and Th17/Treg balances were skewed in peripheral blood of patients with renal cancer towards the Th2 and Th17 profile, respectively, with recruitment of Tregs to the tumour (34). An imbalance in Th17/Treg cells was related to gastric cancer (35) and lung cancer (36). Because immunohistochemically we observed only very low tumour infiltration by CD4⁺ cells (without any clear difference between progressive and SR tumours), CD4⁺ subpopulations probably have only a negligible (if any) effect on rat tumours. The same is also true for CD8⁺ cells. The very low number of these T-cells found in both tumour types suggests that they probably do not significantly affect growth and SR of rat tumours.

In conclusion, subcutaneous inoculation of cancer cells into Lewis rats led to development of progressive and SR tumour or to total suppression of cancer cells. Although this study used an inbred line of rats, this result suggests that there must be differences among the animals in the cells of the immune system or in the expression of cytokines that cause the different development of tumours. Because there was a very low number of CD4⁺ and CD8⁺ cells and high infiltration by CD161⁺ cells, we suppose that NK cells alone play a key role in the destruction of cancer cells during SR of subcutaneous tumours in this Lewis rat sarcoma model. Our finding, suggesting the participation of NK cells in this process of 'natural immunotherapy', supports the idea of utilization of autologous NK cells in adoptive immunotherapy of solid tumours, as already applied in clinical trials (37).

Conflicts of Interests

The Authors declare that they have no conflicts of interests in relation to this study.

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