Basic Research Paper

Observation on myeloid origin of neovascular endothelial cells and infiltration of bone-marrow-originated inflammatory cells in a murine tumor model

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Background and Objective: Some studies indicate that endothelial progenitor cells (EPCs) originated from the bone marrow participate in neoplastic angiogenesis, and that bone marrow origin of inflammatory cells potentially contribute to neoplastic invasion, angiogenesis and metastasis. This study was to observe the origin of neovascular endothelial cells and infiltration of bone marrow-originated inflammatory cells in a murine tumor model. Methods: Healthy C57BL/6 mice were irradiated with 60Co at 8 Gy. Bone marrow cells of green fluorescent protein (GFP) transgenic C57BL/6 mice (donors) were transplanted intravenously into C57BL/6 mice (recipients) via the tail vein 24 h after irradiation. Lewis lung tumor cells were inoculated subcutaneously into recipient mice two weeks after transplantation. The xenograft tumors were removed until their diameters reached approximately 1–2 cm. Subsequently, tumor vessels and inflammatory cells were observed under fluorescent microscopy and detected using immunohistochemistry (IHC). Results: Unsuccessful green fluorescence emitted by neoplastic vascular endothelial cells and inflammatory cells was observed, most of which appeared positive IHC staining. A large number of macrophages were observed inside or adjacent to the necrotic areas of the tumor. A few lymphatic cells were mainly dispersed inside tumor stroma and tumor cells. Conclusions: Partial endothelial cells of neoplastic neo vessels originate from the bone marrow. The murine tumor model could be used as a specific and direct approach to observe bone marrow-originated cells in neoplasms.

Sustainable growth of neoplasm mainly depends on angiogenesis.1 Tumor blood vessels provide oxygen and nutrition necessary for tumor growth and clear away metabolite of tumor cells. Therefore angiogenesis is very important for tumor growth. Most tumor vessels formed by the means of outgrowth from the host vasculature. In 1997, Aashara et al.2 proposed that myeloid origin of endothelial progenitor cells (EPCs) contributed to neoplastic angiogenesis. In this study, in order to clarify myeloid origin of neovascular endothelial cells and observe the infiltration of inflammatory cells in tumor tissues, we used green fluorescent protein (GFP) transgenic C57BL/6 mice (all cells expressing MHC-Ⅰ antigen emitted green fluorescence) as donors to provide bone marrow, and ordinary sanitary C57BL/6 mice as recipients (all cells expressing MHC-Ⅰ antigen did not emit green fluorescence). After bone marrow transplantation, a murine tumor model was established to detect the myeloid origin of neoplastic vascular endothelial cells and the infiltration of bone marrow originated inflammatory cells in tumor tissues, thus to explore a novel approach to observe cells of myeloid origin in tumor tissues.

Materials and Methods

Experimental animals. Fifteen male C57BL/6 inbred mice, aged 8–10 weeks, an average weight of 20 g, were provided by Shanghai SLAC laboratory animal Co. Ltd. ([certificate number: SCXK(HU)2007-0005]. Male transgenic C57BL/6 mice expressing GFP under the control of the MHC-Ⅰ antigen promoter were provided by Chengdu Da Shuo Laboratory Animal Co. Ltd. [certificate number: SCXK (CHUAN) 2004-16]. All mice were bred in the independent vent center (IVC) of the experimental animal center in Henan province.

Preparation and transplantation of bone marrow cells. GFP transgenic C57BL/6 mice were killed by dislocating their cervical vertebra, Mice by sterilized in 2% iodine tincture and 75% alcohol for 3–5 min. Subsequently, femurs and tibias were dissected and the two ends of bones were cut. Bone marrow was ejected using RPMI1640 culture media to prepare cell suspension, which was centrifuged and adjusted to $2 \times 10^7$ /ml. Ordinary sanitary C57BL/6 mice were exposed to systemic irradiation of 60Co at 8 Gy. Twenty-four hours after irradiation, $1 \times 10^7$ (0.5ml) bone marrow cells of GFP transgenic C57BL/6 mice were transplanted intravenously into ordinary C57BL/6 mice via the tail vein. Mice were orally given gentamicin (32 $\times 10^4$ U/L) started from five days before transplantation to two weeks after transplantation to prevent infection.3
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Subcutaneous inoculation of tumor cells. Two weeks after bone marrow transplantation, mice bearing Lewis lung cancer were killed by dislocating their cervical vertebra, and sterilized in 75% alcohol for 5 min. Subsequently, tumor tissues were dissected from the axilla inside a superclean bench. After removing the fibro-membrane and necrotic areas, tumor tissues were pulverized in sterilized plates and ground slowly in the tissue grinder with normal saline (NS) at 4°C to make the cell suspension. The cells number was counted using 4% trypan blue. If the cell viability was greater than 95%, the cell concentration was adjusted to be 5 × 10^6/ml. Then 0.2 ml suspension of tumor cell (1 × 10^6 cells) was innoculated subcutaneously into ordinary C57BL/6 mice.

Observation on blood vessels and inflammatory cells in tumor tissue of recipient mice. On the seventh day after innoculation, mice were anesthetized by peritoneal injection of 10 mg/kg ketamine and 20 mg/kg xylazine when the tumor diameter was approximately 1–2 cm. Then mice were fixed at the resting position, and operation was performed to expose the heart. After opening a small hole at the cardiac apex, a blunt pinhead connecting to a rinse tube was inserted swiftly through the left ventricle up to the ascending aorta. A small hole was opened in the right atrium to discharge blood and NS rinse. Following a rinse with 100–150ml NS, 100–150ml Milloning formaldehyde solution was used to fix tumor tissues. Next, tumor tissues were fixed again in vitro. Pathological sections were made, and tumor vessels and inflammatory cells were observed under fluorescence microscopy.

Antibodies Anti-CD31, CD68, CD3, CD20 antibodies were purchased from Beijing Bo Ao-Sen Biology-Engineering Corporation. Anti-Cy3 antibody was bought from Wuhan Bo Shi-De Biology-Engineering Corporation. Immunohistochemistry (IHC) was performed as follows: after the recovery of antigen, slides were incubated with appropriately diluted first antibodies (CD31, CD68, CD3, CD20) at 4°C overnight, and then with fluorescent secondary antibody Cy3. Finally, after a PBS rinse, slides were observed under microscopy.

Results

After bone marrow transplantation, in tumor tissues of recipient mice, vascular endothelial cells, which emitted green fluorescence and hemocytes were observed under fluorescence microscopy (Fig. 1A). Sporadic green fluorescence emitted by unsuccessfully distributed vascular endothelial cells were observed, suggesting that those endothelial cells were originated from bone marrow. Blood vessels in tumor tissues were twisted with sinusoidal dilation and irregular lumens. The vascular wall was thin, covered by one layer of endothelial cells with incomplete basal membranes, lacking of smooth muscles. Some lumens, which were not covered with endothelial, contained hemocytes emitting green fluorescence (Fig.1B).4

Green fluorescence emitted by inflammatory cells was directly observed under fluorescence microscopy (Fig. 2). Single or clusters of GFP positive cells originated from bone marrow were mostly appeared around tumor cells and necrotic areas. A few inflammatory cells were dispersed inside tumor cells.

Assessed by IHC staining of CD31 and Cy3 (Fig. 3), endothelial cells of neoplastic vessels emitting yellow fluorescence were found to be originated from bone marrow; while those emitting red fluorescence were not, which we presumed that, might be sprouting from host blood vessels. The new blood vessels in tumor tissues were irregular, mainly located at peripheral regions where tumor cells thrived. A small quantity of capillaries appeared inside tumor cells.

Based on results of IHC staining of CD68 and Cy3 (Fig. 4), clusters of macrophages were found emitting red fluorescence mainly in necrotic regions, some of which gathered together to form multinucleated giant cells after phagocytozing dead tumor cells (Fig. 4A). A few single macrophages were dispersed around or inside tumor cells (Fig. 4B).

Findings from IHC staining of CD3, CD20 and Cy3 (Fig. 5) revealed that singer of clusters of T cells and B cells were mainly distributed in tumor stroma and the junction area of tumor cells. A few small lymphocytes were dispersed inside tumor cells.

Discussion

Neoplastic angiogenesis plays an important role in carcinogenesis and development of cancer. In 1997, Asahara et al.2 extracted EPCs from human peripheral blood using cell surface antigen CD34 and suggested that EPCs contribute to neoplastic angiogenesis. EPCs have capacity to migrate, proliferate, differentiate into endothelial cells lines, and balance vascular metabolism. In addition, studies show that carcinogenesis is partially associated with chronic inflammation, which can promote angiogenesis, proliferation and metastasis of tumor cells. Thereby, it is proposed that inhibition of neoplastic...
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mainly infiltrate in tumor necrotic areas, presumably attributed to inflammatory reaction caused by necrosis. A large number of macrophages were activated to gather around necrotic areas to phagocytize dead cells, some of which were even transformed to multinucleated giant cells to enhance specific immunity. A scarcity of macrophages was dispersed in the peripheral region of tumor cells, probably due to immunological reaction caused by tumor cell antigen. Besides, Geraidine et al. found accumulation of labeled macrophages in tumor tissues when they were transfused into the animal model. This reveals that macrophages possess chemotactic capacity. Lymphocytes were rarely seen among inflammatory cells and mainly distributed in tumor stroma and the junction of tumor cells, a few of which were dispersed inside tumor cells. This is presumably due to the antigen-antibody reaction. Some studies show that inflammatory cells build a comfortable environment where blood vessels and lymphatic vessels could thrive. Moreover, by regulating the expression of chemokines, tumor cells can promote growth, progression and metastasis of themselves. Better understanding of inflammatory cells may pave a way to explore anti-tumor drugs targeting inflammation, such as tumor necrosis factor (TNF) inhibitors, chemokine antagonists, nonsteroidal anti-inflammatory drugs.

The murine tumor model established in this study makes it possible to directly observe blood vessels and inflammatory cells in tumor tissues under fluorescence microscopy. Moreover, three-dimensional structures of blood vessels could be observed under confocal microscopy. Thus, this animal model can be used as a tool for studying angiogenesis.
feasible method to observe cells originating from bone marrow in tumor tissues.

Based on this study, we will investigate cytotoxicity of metronomic chemotherapy on tumor blood vessels and the anti-angiogenesis effect of anti-inflammation, so as to find out the optimal dosage and approach of metronomic chemotherapy.

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References