Microvessel Origin and Distribution in Pulmonary Metastases of B16 Melanoma: Implication for Adoptive Immunotherapy

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ABSTRACT

To elucidate the role of tumor vascularization on the localization of adoptively transferred, interleukin 2-activated natural killer (A-NK) cells, pulmonary B16 melanoma metastases were analyzed with respect to location, morphological appearance, origin and density of microvessels, and infiltration by A-NK cells. The B16 melanoma metastases could be divided into four subtypes according to their location (superficial or deep in the lung parenchyma) and morphological appearance (compact or loose). Localization of adoptively transferred A-NK cells into the four subtypes of B16 pulmonary metastases differed significantly. More than 800 A-NK cells/mm² were found in metastases of the deep-loose type, compared to approximately 400/mm² A-NK cells in the superficial-loose subtypes of B16 pulmonary metastases. Differences in the density of A-NK cells in the metastatic subtypes correlated with the number of A-NK cells that localized into these metastases. The resistance of metastases of different origins to the vascular supply of metastases in the lungs can arise from the heterogeneous infiltration of pulmonary metastases by A-NK cells and to the origin of their blood supply. LM, FM, and ThM were used to locate routes for A-NK cells to infiltrate melanoma metastases and to the origin of their blood supply. The mechanism(s) behind the antitumor effect exerted by adoptively transferred effector cells might explain, in part, why adoptive immunotherapy seldom results in complete eradication of disseminated cancer.

INTRODUCTION

AIT⁴ is based on the observations that lymphocytes, activated ex vivo by IL-2, proliferate vigorously and at the same time develop into very potent killer cells, capable of lysing a large variety of malignant cells while sparing normal cells (1). Upon injection, the effector cells are expected to localize and infiltrate malignant tissues and subsequently kill all malignant cells by contact-mediated lysis. The many reports on successful AIT of cancer in animal models (2–6) fully justified the fast transformation of this therapeutic modality to clinical trials, but the results of these trials have, unfortunately, far from fulfilled the expectations. It is, however, important to note that some patients have enjoyed a long-lasting complete response to such treatment. The challenge is to better understand why such impressive results occur in only a few patients and how to make such results occur more consistently and with higher frequency.

The mechanism(s) behind the antitumor effect exerted by adoptively transferred effector cells is not yet understood, but several possibilities exist. Once in the tumor tissue, the effector cells may interact directly with and kill tumor cells via cell-cell contact, or effector cells may release different substances causing tumor cell damage and/or microvascular coagulopathy (6). Alternatively, the effector cells might damage or activate the tumor microvasculature, leading to extravasation of both exo- and endogenous effector cells and/or their potentially cytotoxic products, such as IL-2, tumor necrosis factor α, transforming growth factor β, and IFN-γ (7, 8).

Studies by Basse et al. (9, 10) and Kuppen et al. (11) have shown that lymphokine A-NK cells are able to localize into metastases of different origins. In the tumors, the A-NK cells established close contact with both tumor cells and tumor endothelial cells (10). Although the tumor localization was very specific, only 5–10% of the injected cells reached the malignant tissues. The density of A-NK cells in individual tumors varied considerably, and virtually no A-NK cells were seen in 5–10% of the lesions (9). Furthermore, substantial localization of A-NK cells in liver metastases was only seen following locoregional administration of the killer cells into the portal vein (9). Likewise, few A-NK cells injected by the i.v. route were found in metastases in the peritoneal cavity and the s.c. tissue, but significant infiltration of these tumors was observed if the cells were injected locoregionally into the peritoneal cavity and the s.c. tissue around the tumors, respectively.⁴ These observations indicate that the local administration of A-NK cells is more efficient for infiltration and might be a prerequisite for their accumulation in malignant tissue.

With respect to metastases in the lungs, i.v. injection can be considered as a local route of injection, since the lung microcirculation represents the first capillary bed the killer cells encounter. However, the vascular supply of metastases in the lungs can arise from both the pulmonary and the bronchial arteries (12, 13). This means that following i.v. injection, the killer cells might reach only some of the metastases directly, i.e., those receiving blood from the pulmonary arteries. To reach metastases nourished by the bronchial arteries, the killer cells must first pass the pulmonary microcirculation, return to the left ventricle of the heart, and then enter the bronchial arteries. Since the percentage of the cardiac output delivered to the bronchial arteries is less than 5%, the number of effector cells entering these vessels is limited.

The present study, we hypothesize that part of the explanation for the heterogeneous infiltration of pulmonary metastases by A-NK cells might be related to variations in the blood supply to these metastases and to the origin of their blood supply. LM, FM, and TEM were used to define the distribution and connection of preexisting as well as newly formed vessels in relation to B16 metastases in mouse lungs and to locate routes for A-NK cells to infiltrate melanoma metastases in this organ.

MATERIALS AND METHODS

Animals and Anesthesia. Ten-to-12-week old female mice of the C57Bl/6 strain were used. Mice were anesthetized by i.p. injections of sodium pentobarbital (10 mg/100g body weight).

Malignoma Cell Preparation. The tumor cell line used in the study was the B16-F1 melanoma. The cells were maintained in vitro in RPMI 1640 supplemented with 2% FCS, 2 mM glutamine, 0.8 g/l streptomycin, and 1.6 × 10^5 units/liter penicillin. Adherent cells were detached by exposure to 0.02% EDTA in PBS for 5–8 min and washed three times in PBS. The cells were then resuspended in RPMI 1640 and adjusted to a final concentration of 1.67 × 10^6 cells/ml. The cell viability always exceeded 95%, as judged by trypan blue dye exclusion test.

Pulmonary melanoma metastases were established in C57Bl/6 mice by injection of 0.5 × 10^6 cells in 0.3 ml medium in the lateral tail vein. The lungs were removed at days 10–12 and processed for LM, FM, and TEM as described below.

Preparation of A-NK Cells. A-NK cells were prepared as described earlier by Gunji et al. (14). Briefly, mouse spleens were harvested, and a single-cell suspension was prepared in RPMI 1640. Erythrocytes were lysed by incubation with ammonium chloride-potassium buffer, and the cells were subsequently washed twice in RPMI 1640. The cells were then transferred to T150 flasks and cultured (37°C with 5% CO_2) in 50 ml of complete medium (CM) consisting of RPMI 1640 with 10% FCS, 2-mercaptopethanol (5 × 10^-3 M), nonessential amino acids, and antibiotics. The CM was supplemented with highly purified recombinant human IL-2 (1000 units/ml), kindly provided by the Chiron Corp., Emeryville, CA; Refs. 15 and 16). After 2–3 days of incubation, nonadherent cells were removed, and the flasks were gently washed with prewarmed (37°C) CM to remove cells not firmly attached to the plastic. Fresh CM (50 ml), supplemented as described above, was added, and the cells were cultured for an additional 2–3 days. After a total of 5 days of culturing, cells were harvested after a short treatment with 0.02% EDTA; then the cells were washed twice with RPMI 1640 before use.

Labeling of A-NK Cells with Rhodamine. A-NK cells were labeled with rhodamine as described previously (9, 17). Briefly, 3–6 × 10^6 A-NK cells were incubated with 15 μg rhodamine (TRITC, Sigma) in 50 ml RPMI 1640 for 30 min at 37°C. After labeling, the cells were washed twice in RPMI 1640 and adjusted to appropriate concentrations.

Adoptive Transfer of A-NK Cells. On day 12 of tumor growth, each mouse was palpated for 0.3 ml RPMI 1640 containing approximately 2 × 10^7 rhodamine-labeled A-NK cells (specimens for TEM examination were given unlabeled cells) into the lateral tail vein. Mice received 25,000 Cetus units IL-2 in 0.5 ml RPMI 1640 at 0, 4, 8, and 12 h following injection of the A-NK cells.

Experimental Procedure (Lanthanum Perfusion). At the time of the experiments, the animals were anesthetized i.p., and a thoracotomy was performed. The lung lobes were then inspected for melanoma metastases. An intraventricular catheter was inserted, either in the right or the left ventricle of the heart for perfusion of the pulmonary and bronchial arteries, respectively, and a preperfusion (TRIS-Tyrode solution supplemented with 1% BSA) of the lung circulation was performed for 3 min to empty the vessels of erythrocytes and leukocytes. The preperfusion was then followed by a bolus of 1% lanthanum chloride infused during 15 s to ultrastructurally label microvessels supplied by the respective vascular pathways. After the La**+** bolus, TRIS-Tyrode-BSA was infused for 30 s to remove nonbound lanthanum. The lungs were then fixed by both intratracheal and intravascular fixation with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 5 min. Lungs with adoptively transferred A-NK cells were taken at 16 h after injection and were fixed in the same way as described above (La**+** perfusion was excluded).

TEM. After vascular and intratracheal fixation, the lungs were kept in glutaraldehyde overnight, and biopsies of melanoma metastases with surrounding normal lung tissue were taken out and postfixed in 1% OsO_4 in 0.1 M sodium cacodylate. The specimens were then stained en bloc with uranyl acetate, dehydrated, and infiltrated with Agar 100 epoxy resin. Ultrathin sections were cut in a Reichert Ultracut E microtome, stained with lead citrate, and examined in a Zeiss CEM 902 electron microscope equipped with an integrated electron energy loss spectrometer allowing element-specific imaging.

LM. Lungs were postfixed overnight in 2.5% glutaraldehyde and were dehydrated and infiltrated with Agar 100. Ten 1-μm sections from each of four animals were cut and stained with hematoxylin and eosin. Microvessels were identified (×100 oil immersion objective), either by the presence of erythrocytes or by the endothelial lining (in the case of empty vessels). In each of the 10 sections, the area of microvessels was measured in 3 randomly chosen fields (area = 0.03 mm^2) of metastatic (superficial-loose, deep-loose, and compact) and normal lung tissue using a Nikon FXA photomicroscope connected to image analysis equipment (Freeland, Sight Systems, Newport, England). The mean vessel area was calculated as: \( V_d = V/T \times 100 \), where \( V \) is the vessel, \( T \) is total tissue, \( a \) is area, and \( d \) is density. The mean density of cells/mm^2, \( C_d \), of individual B16 metastases was estimated by counting the number of nuclei (N) in three randomly chosen areas (measuring \( 7 \times 10^{-4} \) mm^2) of eight loose and eight compact B16 metastases from three different animals:

\[ C_d = (N_1 + N_2 + N_3) \times \left( \frac{3 \times 7 \times 10^{-4}}{0.01} \right) \]

where 0.01 is the section thickness in mm.

RESULTS

Classification of 10–12-Day-Old B16 Melanoma Metastases. The pulmonary B16 melanoma metastases could be classified in four subgroups by means of their location and ultrastructural appearance (Fig. 1):

(a) Superficial-loose metastases were located just beneath the mesothelium, separating the lung parenchyma from the mesothelium (Fig. 1d). These metastases spread out under the mesothelium in a discoid shape. Although the diameter of these metastases (as seen from the surface of the lungs) was often larger than 300 μm (equal to 15–20 cell diameters), their thickness seldom exceeded 3–6 cell layers. The superficial-loose metastases seemed to respect the mesothelial border, but their demarcation to the underlying parenchyma was irregular. More than 90% of these metastases were pigmented, but individual tumor cells contained various amounts of melanin. The melanin within individual cells was distributed in a heterogeneous pattern, sometimes evenly distributed in the cytoplasm, sometimes appearing in 1–2 densely packed clumps. The shape of the melanoma cells in these metastases varied considerably.

(b) Superficial-compact metastases were also growing on the surface of the lungs but formed spherical nodules, often protruding through the mesothelial lining (Fig. 1d). The majority of the mela-
tumor cells in these metastases were of the same spherical or ellipsoid shape containing almost the same amount of melanin evenly distributed in the cytoplasm. These compact metastases appeared to be composed of tumor cells only, containing no other cell types and very little extracellular tissue. These metastases formed clear lines of demarcation to the surrounding normal tissue.

(c) Deep-loose metastases were found deeper in the lung tissue, surrounded by normal tissue on all sides (Fig. 1b). The melanoma cells in these metastases grew along the alveolar walls as well as in the alveolar spaces. The deep-loose metastases often appeared spherical in shape, but their demarcation to the surrounding tissue was poorly defined. The melanin in these metastases was heterogeneously distributed, and the shape of the cells varied considerably as in the superficial-loose metastases. Alveolar cells, lymphocytes, and granulocytes were found in these metastases, and various amounts of extracellular tissue were seen in between the melanoma cells.

(d) Deep-compact metastases resembled the superficial-compact metastases in that they had a distinct line of demarcation to the surrounding tissue and that they grew in an expansive fashion, apparently without invading the surrounding tissue or alveolar spaces (Fig. 1b). As in the superficial-compact metastases, the melanoma cells in the deep-compact metastases were mainly of spherical or ellipsoid shape containing almost the same amount of melanin, evenly distributed throughout the cytoplasm. Interestingly, these metastases were mostly found growing in a sleeve-like pattern around large tubular structures, such as arterioles and bronchioles. On cross-sections, these metastases appeared bigger than the deep-loose metastases, indicating a high growth potential.

Comparison of density of cells in eight compact (superficial and deep) and eight loose (superficial and deep) metastases revealed that the compact type of metastases contained approximately 15% more cells than the loose-type metastases (1.08 \( \times 10^6 \) ± 0.11 \( \times 10^6 \) cells/mm\(^3\) and 0.95 \( \times 10^6 \) ± 0.14 \( \times 10^6 \) cells/mm\(^3\), respectively). The density ranged from 0.90 \( \times 10^6 \) to 1.24 \( \times 10^6 \) cells/mm\(^3\) in the compact metastases and from 0.71 \( \times 10^6 \) to 1.13 \( \times 10^6 \) cells/mm\(^3\) in the loose type of metastases, i.e., some of the loose metastases contained more cells/mm\(^3\) than some of the compact metastases.

**Microvessel Distribution and Connections (TEM, LM).** To analyze the distribution of microvessels in the four types of pulmonary B16 metastases, sections of lung tissue were examined by TEM following perfusion of the lungs with 1% lanthanum chloride installed via the right ventricle (for perfusion of the pulmonary circulation) or the left ventricle (for perfusion of the bronchial arteries) of the heart, as described in “Materials and Methods.” The mean vessel density in the metastatic tissue was determined by light microscopy.

Superficial-loose metastases, located just beneath the mesothelium, were as a rule avascular, but in the border between normal lung tissue and tumor tissue, there were high numbers of preexisting alveolar capillaries with typical morphology for gas exchange vessels. These preexisting microvessels were always labeled with La\(^{++}\) (Fig. 2a).
TUMOR MICROCIRCULATION AND ADOPTIVE IMMUNOTHERAPY

Fig. 2. Electron microscopy of B16 metastases. a, low power electron micrograph of a small superficial melanoma metastasis. The heavily pigmented melanoma cells are located immediately beneath the mesothelial lining (M) but do not penetrate the submesothelial basal lamina. Note also that one melanoma cell (arrowhead) is in the process of invading an alveolus (Alv). *, an alveolar capillary on the deep aspect of the tumor labeled with lanthanum after pulmonary administration. Bar, 10 μm. b, pulmonary artery perfusion (via the right ventricle of the heart) labeled vascular channels with a varying degree of patency in the central part of a deep-loose metastasis. Some label increased the contrast of the extravascular interstitial space (arrowhead), indicating leakage. Vessel lumina (*) and RBC (R) are indicated. Bar, 5 μm. c, appearance of nonpatent vascular channels in a deep-loose metastasis distinctly labeled with lanthanum via the pulmonary route. These structures probably represent capillary sprouts. Bar, 5 μm. d, deep-loose metastasis. A probable capillary sprout (arrowheads), the thick periendothelial basal lamina) of bronchial vessel origin; note scattered lanthanum deposits along the convoluted lumen. Bar, 2.5 μm. e, deep-loose metastasis. Patent capillary containing a RBC and with more mature appearance than the sprout in d labeled with scattered lanthanum after perfusion of bronchial vessels via the left ventricle of the heart. Bar, 2.5 μm. f, an elongated A-NK cell making cell-cell contacts with melanoma cells inside a deep-loose metastasis. Arrowheads, the A-NK cell specific dual-compartment granules. Bar, 2.5 μm.

introduced via the pulmonary arteries, but no labeling could be visualized after bronchial installation of La⁺⁺⁺. On very rare occasions, labeled vessels could be seen in the periphery of the tumor tissue on the mesothelial side.

Deep-loose metastases were located in the lung parenchyma and were always surrounded by normal alveolar tissue. Capillaries within this tissue, adjacent to the tumor, showed lanthanum labeling after pulmonary administration (Fig. 2b), and the tumor interdigitated with these capillaries, depending upon the degree of tumor demarcation and fibroblast pseudocapside formation. Within the tumor, high numbers of nonpatent, collapsed vascular channels were seen, having the morphology of probable capillary sprouts (Fig. 2c). In tumor vessels, lanthanum leaked into the interstitium to a varying degree (Fig. 2b). It was also noticed that in some vascular channels, melanoma cells formed parts of the channel wall. A few microvessels with bronchial connections were observed, both with capillary sprout appearance (Fig. 2d) and more mature morphology (Fig. 2e).

Superficial- and deep-compact metastases apparently lacked intratumoral microvessels, since no lanthanum could be visualized inside these metastases. The deep-compact metastases often grew around arterioles and bronchioles but never broke their basal membrane or invaded the lumen.

LM examinations of the vessel density in different metastases revealed that 0.6% area of superficial-loose metastases and 1.3% area
of deep-loose metastases consisted of vessels. Approximately 5—10% of the metastases were classified as "compact" (superficial and deep), and no vessels could be detected in these lesions. The data are summarized in Fig. 4.

**Infiltration of B16 Pulmonary Metastases by A-NK Cells (FM).** The ability of adoptively transferred A-NK cells, labeled with rhodamine, to infiltrate the different types of B16 pulmonary metastases was analyzed by FM. At 16 h following the injection of 20 million A-NK cells into 3 C57BL/6 mice, the number of infiltrating A-NK cells was determined in 5—10 randomly selected metastases of each type (compact, superficial loose, and deep loose) for each animal (Fig. 1, a and c; Fig. 3). A significant difference was seen in the average density of A-NK cells in compact (170 ± 26 cells/mm², mean ± SE), superficial loose (403 ± 27 cells/mm²), and deep loose (795 ± 42 cells/mm²) metastases (Fig. 4). These differences could not be attributed to chance selection of the metastases that were counted (P = .0001; ANOVA treating animals as a fixed factor) nor to chance selection of unusual animals (P = .002, ANOVA treating animals as a random factor). Further, the observed pattern was completely consistent for each animal (Fig. 3). Pairwise comparisons of the infiltration of metastases of the three types within each of the three animals were all significant (P < .001), with the exception of the comparison of compact and superficial loose metastases in the second animal (P = .07). The relative infiltration of A-NK cells into compact, superficial-loose, and deep-loose metastases completely paralleled their vascular density, determined in experiments with different animals (Fig. 4). This suggests that a correlation might exist between A-NK cell infiltration and vascularization of the metastases.

**A-NK Cell Infiltration in Pulmonary Metastases (TEM).** In the superficial-loose metastases, A-NK cells engaged the preexisting microvessels in large numbers and were also seen in partial or complete migration from the capillaries to the interstitium, making cell-cell contact with the melanoma cells. In the deep-loose metastases, A-NK cells were seen in the same positions as in the superficial metastases, but A-NK cells were also seen engaging the vascular channels, capillary sprouts, and also in various grades of cell-cell contact with the melanoma cells (Fig. 2f). In the compact types of metastases, very few A-NK cells (<200/mm²) were found inside the metastases or in the border zone to the normal lung tissue (Fig. 1).

**DISCUSSION**

The efficacy of AIT might depend on the number of adoptively transferred killer cells localizing to the malignant tissues. It is, therefore, of interest to identify the conditions leading to optimal lodging of killer cells in tumors and tumor metastases. Although we have demonstrated previously that adoptively transferred A-NK cells are able to localize specifically into pulmonary metastases (9—11), it is still unknown exactly how these cells reach the malignant tissues. In the present study, we have analyzed the ability of A-NK cells to localize into B16 lung metastases according to their location (superficial or deep) and histological appearance (compact or loose). We have also studied the origin and distribution of vessels in the different types of B16 metastases to identify routes for A-NK cells to infiltrate the malignant tissues.

The average density of A-NK cells in the compact, superficial-, and deep-loose metastases correlated well with the vascular density of these types of metastases. This indicates that vascular access may be an important factor in primary tumor localization by A-NK cells. Since lanthanum perfused via the bronchial arteries labeled neither the compact nor the superficial-loose metastases, the low degree of infiltration in these types of metastases cannot be explained by preferential vascularization from the bronchial circulation. In the deep-loose metastases, connections with the bronchial vasculature were confirmed at later time points, but these vessels cannot be expected to contribute significantly to infiltration for two reasons: (a) the percentage of cardiac output going to the bronchial arteries is very low (<5%); and (b) the effector cells recirculate very inefficiently after i.v. administration (9, 18). Therefore, a metastasis that is nourished predominantly or solely by bronchial blood flow would most certainly escape immediate A-NK infiltration. Such a situation might develop in large and old metastases that have attracted angiogenesis selectively from bronchial vessels, as in 16% of experimental metastases analyzed by Milne et al. (19). In our present material, it was not possible to demonstrate such lesions.

The finding that about 5—10% of the total number of the B16 metastases (i.e., metastases of the compact type) were resistant to infiltration by A-NK cells may be explained, in part, by the absence of microvessels in this type of metastases. Since these metastases in general grow around larger vessels, i.e., arterioles, they will probably be nourished from the large vessels and not from the microvasculature per se. This is consistent with the findings by Aliño et al. (20), who found the same pattern of tumor growth around large vessels (and airways) using a Lewis lung carcinoma, and they could also verify the
lack of vascular perfusion in these metastases using the fluorescent dye Hoechst 33342 as a vascular space marker. The possibility that the A-NK cells could get access to the malignant tissue directly from the dye Hoechst 33342 as a vascular space marker. The possibility that the lack of vascular perfusion in these metastases using the fluorescent dye Hoechst 33342 with tumor cells, yet others had migrated totally from the vessels and arterioles occurs very infrequently (21).

The migration of A-NK cells into vascular spheroids in vitro has been observed by Iwasaki (23) and Jääskelainen (24), and A-NK cells administered peritumorally in mouse models of s.c. head and neck tumors (25) and solid intraperitoneal malignancies* migrate deeply into the tumor masses, indicating that the ability of the killer cells to spread inside a tumor might not only depend on the existence of intratumoral vascularization. Thus, the absence of A-NK cells in the B16 melanoma metastases of the compact type might not solely be explained in terms of their lack of microvessels. Other, yet unknown, factors of importance for the intratumoral migration of A-NK cells, such as extracellular matrix proteins and/or chemoattractive substances, might be absent in these metastases.

In conclusion, the variety of localization of A-NK cells into the different types of B16 pulmonary metastases correlates with the vascular density in these tumors, and the majority of the intratumoral microvessels seem to be connected with the pulmonary, rather than the bronchial, arteries. Thus, a blood supply derived mainly from the bronchial arteries could not explain the lower localization of effector cells into metastases of the compact and superficial-loose types compared to the deep-loose type. This existence of micrometastases resistant to infiltration by, for example, A-NK cells, might explain why AIT has seldom resulted in complete eradication of metastases in both rodent models and humans.

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