Lymphoid metastasis of rat My2/De leukemia

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A B S T R A C T
By grafting spontaneous leukemia tumor cells, the myeloid My2/De leukemia rat model was established.
Death was caused by impaired functions of heavily infiltrated organs. In vitro culturing of tumor cells, blood and bone marrow counts and cytochemical reactions indicated the leukemic origin resembling human myeloblastic leukemia. Metastatic spread was followed after i.v. and i.p. injection, and by implantation of leukemia cells under the renal capsule of rats. Primary tumor and metastasis formation was visualized by 18FDG or 11C-methionine administration and MiniPET. The accumulation of radiotracers was measured in different organs and expressed as Differential Absorption Ratios (DARs). Subrenal implantation of My2/De cells resulted in their appearance in other abdominal organs and in parathyric lymph nodes. The release of tumor cells from the primary kidney to the peritoneum was mimicked by the i.p. administration of ink particles. Ink particles deposited in the abdominal organs and in the thoracic lymph nodes, preferentially in parathyric lymph nodes, confirming the notion of lymphatic spread of metastasis.

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1. Introduction

Compared to murine and chicken experiments, spontaneous leukemias in rats are rare. Higher frequency and transmissibility have been described in old rats [1,2]. Rat leukemia models have been established similarly to murine models by viral infection [3–6], by irradiation [7,8] and chemical carcinogenesis [9–12]. Classification of rat leukemias similar to the human FAB system does not exist, nevertheless their cytochemical reactions resemble to human leukemia. Rat models have been used to mimic human acute myelocytic leukemia (AML) [13,14], as these models exhibited a high degree of similarity to human AML [15–18].

Myelomonoblastic leukemia (My1) in rats was induced by chemical carcinogenesis and converted to My1/De cell line [11,19]. Another myeloblastic leukemia named My2 developed spontaneously in 1997 was clearly distinguishable from My1/De. My1 was growing faster and grafted in 2 weeks, the integration of My2 into the host tissue took 4 weeks. My1 caused primarily the enlargement of liver, My2 also the expansion of spleen.

Major aims of this work include: (a) inbread Long–Evans rats for the implantation of myeloid My2/De leukemia cells to observe their in vivo propagation, (b) to culture My2/De cells in vitro and prove their leukemic origin by cytochemical reactions, (c) to distinguish between the hematogenous and lymphatic spread of My2/De cells after their implantation in rats, and (d) to follow the metastatic spread by radiotracers and mimic the metastasis by the subrenal and i.p. implantation of India ink.

2. Materials and methods

2.1. Animals

Inbread Long Evans (LE) rats were kept in a conventional laboratory environment and fed on a semi-synthetic diet (Charles-River, Mo, Kft, Godollo, Hungary) and tap water ad libitum. Experiments followed the 3Rs principle and the criteria outlined in the UK “Guide for the Care and Use of Laboratory Animals” [20], authorized by the Ethical Committee for Animal Research, University of Debrecen.

2.2. Spontaneous leukemia

My2 leukemia was observed in 1997 in a Long Evans male rat of extremely enlarged spleen (4g), high (1.04 × 107/μl) white blood cell number packed with unidentified blast cells. The leukemic blood was injected in control rats (0.5 ml) that became leukemic within one month with a high white blood cell number (1.9–2.8 × 106/μl). Leukemic cells have been maintained by yearly two to three implantations of 10–20 mg spleen slices of leukemic rats under the kidney capsule and storing the enlarged spleen tumor in a Kelvinator at −80 °C. This tumor was distinguished from myeloblastic leukemia named My1/De [11] and named My2/De.
My2/De abbreviation refers to Myelomonocytic leukemia 2 isolated at the University of Debrecen.

### 2.3. Primary cell culture

My2 leukemic rats with at least $6 \times 10^6/\mu l$ white cell number were euthanized with Nembutal, their thigh bones removed, cleared of other tissues and their ends cut off. The content of the resulting femoral tube was removed and washed with saline. The bone marrow was homogenized by pipetting, centrifuged for 5 min at 800 $\times$ g. The pellet was resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. After overnight incubation at 37°C in a 5% CO$_2$ atmosphere, adherent cells were discarded and nonadherent suspension cells subcultured for 3 days. After 4 days the suspension culture differentiated into macrophages.

### 2.4. Surgical implantation of leukemia cells

My2/De cells were implanted under the capsule of the left kidney to follow metastatic leukemia tumor spread. This was performed by placing $10^6$ My2/De cells in 10 $\mu l$ saline on Gelason disc. Animals were anesthetized by i.p. administration of 3 mg/100 g pentobarbital (Nembutal, Ceva-Phyllaxia Rt. Budapest, Hungary). The tumor cell-containing disc or lymph node was placed under the renal capsule of the left kidney. Stitches were put in the wound. Four weeks after implantation myelomonocytic leukemia developed. Leukemic rats died after 5 weeks. Animals were euthanized before dissection by an i.p. 300 mg/kg overdose of Nembutal.

### 2.5. Measurements of $^{18}$FDG and $^{11}$C-methionine accumulation

#### 2.5.1. Radiotracer synthesis

The glucose analog $2-[^{18}$F]fluoro-2-deoxy-D-glucose ($^{18}$FDG) was synthesized and labeled with the positron-decay isotope $^{18}$F according to Hamacher [21]. $^{11}$C-Methionine was synthesized by the modified method of Ishiwata [22]. Radiotracers were synthesized in the Department of Nuclear Medicine, University of Debrecen.

#### 2.5.2. In vitro radiotracer accumulation

$^{18}$FDG and $^{11}$C-Methionine (MET) were used as tracers to measure accumulation in control and My2/De cells. Bone marrow cells (10$^6$) obtained from control and leukemic rats after 4 weeks of implantation were preincubated in PBS containing 5 mM D-glucose at 37°C for 10 min. Preincubation was followed by the addition of 0.37 MBq/ml $^{18}$FDG or 1.85 MBq/ml $^{11}$C-Methionine for the time indicated in the experiments. Uptake of radiopharmacorn was terminated by the addition of ice cold PBS. Cells were washed three times with cold PBS. The accumulation of radiotracer was measured for 1 min in gamma counter (Canberra, Packard). Uptake was corrected for disintegration and given in counts per min (cpm) units/10$^6$ cells. The tracer accumulation in 10$^6$ cells was also expressed as the percentage of the incubating dose.

#### 2.5.3. Tissue distribution of radiotracers

$^{18}$FDG and $^{11}$C-Methionine were used to measure their accumulation in tissues. One month after tumor implantation 11.1 $\pm$ 1.5 MBq $^{18}$FDG or $^{11}$C-Methionine were injected via the tail vein into the control and tumor-bearing rats. 30 min after $^{11}$C-Methionine and 50 min after $^{18}$FDG injections animals were euthanized. Blood was taken PTNs. The weight and radioactivity of tissue samples were determined and values expressed as Differential Absorption Ratios (DARs). DAR was calculated as: (accumulated radioactivity/g tissue)/accumulated radioactivity/g tissue).

#### 2.5.4. MiniPET examinations

One month after tumor cell implantation rats were injected via the tail vein with 11.1 $\pm$ 1.5 MBq $^{18}$FDG or $^{11}$C-Methionine. 30 min after $^{11}$C-Methionine and 50 min after $^{18}$FDG tracer injection animals were anesthetized. 20-min static single-frame PET scans were performed using our small animal MiniPET-II scanner to visualize the tumors.

#### 2.5.5. PET data analysis

Radiotracer uptake was expressed as standardized uptake value (SUV). Ellipsoidal 3-dimensional regions of interest (ROI) were manually drawn around the edge of the tumor xenograft activities by visual inspection using the BrainCad software (http://www.minipetc.hu). The standardized uptake value (SUVR) was calculated as:

$$SUVR = \frac{ROI \text{ activity (Bq/ml)} \times \text{ injected activity (Bq/animal weight (g))}}{2.63},$$

assuming a density of 1 g/cm$^3$.

### 2.6. Metastatic studies

The aim of these experiments was to define where the subrenally implanted cells metastasize. The localization of the first metastasis was studied by implanting $10^6$ My2/De cells under the subrenal capsule of three male and three female rats. Animals were euthanized 28 days (2 each, one male and one female) after tumor cell implantation, their parathymic lymph nodes were removed. The reimplantation of tumorous parathymic glands under the renal capsule was. The maintainance of My2/De cells by the reimplantation of the tumorous PTNs was reproducible. To mimic tumor spread, 0.5 ml India ink (Gunther Wagner, Pelikan Werke, Hannover, Germany) suspension was injected i.v. or administered intraperitoneally.

#### 2.7. Microscopic staining

Samples were taken from organs of control and My2/De leukemic rats. Cells either were mounted on slides dried and stained with Giemsa for blood, Methanol-Giemsa for spleen, or May–Grünewald–Giemsa solution for PTN cells. Differential cytochemical staining included myeloperoxidase, Sudan Black B, acid phosphatase (AP) and a-naphthyl-acetate (ANA) reactions.

#### 2.7.1. Myeloperoxidase (MPO) assay

We have used Hanker's myeloperoxidase assay that is negative in lymphocytes and megakaryocytes, but shows fine brown-black granularity in monocytes [23]. Reagents were obtained from Sigma–Aldrich, Hungary (Budapest) and the reaction was carried out as described by the protocol.

#### 2.7.2. Sudan Black B staining

Sudan Black B staining is similar to the myeloperoxidase reaction, able to distinguish between acute lymphocytic leukemia and acute myelogenous leukemia. We have used the modified Sheehan-Storey method [24] using the reagents and protocol of Sigma–Aldrich (Budapest, Hungary).

#### 2.7.3. Acid phosphatase (AP) reaction

We have used the method of Davis and Omstein [25] that is carried out in an acidic environment (pH 5.5) and hydrolyses naphthyl phosphate to naphthol and phosphoric acid producing a red-brown compound in the presence of basic fuchsin. The reagents and the protocol were provided by Sigma–Aldrich.

#### 2.7.4. a-Naphthyl-acetate (ANA) reaction

This cytochemical reaction is able to distinguish between cells of the mono- and neutrophil lineages. The leukocyte esterase hydrolyses the substrate and the released naphthol couples with the diazonium salt resulting in a red-brown or black-brown precipitated ring [26]. Reagents were purchased from Sigma–Aldrich. Cytochemical reactions are given in Table 1.

### 3. Results

#### 3.1. Subrenal implantation of My2/De cells

Four Long–Evans rats (2 males, 2 females) were subjected to implantation of tumor cells ($10^6$ cells) under the subrenal capsule (Fig. 1A). After 5 weeks animals were euthanized, sectioned and smeared from bone marrow, spleen and parathymic lymph nodes were prepared. Autopsy findings were uniform, significant differences in the masses of different organs occurred (Table 2). Largest proportion of weight increase (40–70%) was observed in...
parathythymic lymph nodes that contain B-lymphoblasts migrating from the bone marrow to these tiny glands (Table 2). The tumorous PTNs occupied a large portion of the thorax (Fig. 1B and C). The second largest weight increase was measured in spleen (~5.3×), followed by the liver (~1.8×) and the kidney (~1.2×) (Table 2; Fig. 1B and C).

3.2. Tracing tumor formation in rats by miniPET

This series of experiments visualized primary tumor and metastasis formation in rats that were implanted My2/De cells under the capsule of left kidney by SRCA. Four weeks after My2/De implantation rats were anesthetized and injected with 11.1 ± 1.5 MBq 18FDG or 11C-methionine via the tail vein. After 50 min static 18FDG-PET imaging was performed (Fig. 1D) (Fig. 1D upper and lower panel). PET imaging was done 30 min after injecting 11C-methionine into the caudal vein (Fig. 1E upper and lower panel). Exposures were taken in the coronal planes. The analysis of these images proved that the growing tumors projected metastases to the PTNs. The SUVmean value of parathythymic lymph nodes after the injection of 18FDG was 2.92 ± 0.5 and after 11C-methionine injection 3.43 ± 0.6. These values are ~4.7 and ~5.5 times higher, respectively than corresponding SUVmean values of the control parathythymic lymph nodes.

These findings were supported by the accumulation of 18FDG expressed as Differential Absorption Ratio (DAR). The DAR was significantly higher in the tumor, spleen, liver, left kidney, than in the same organs of the control rats. Highest 18FDG-DAR value was obtained in the PTN of My2/De leukemic rats (Fig. 2A). Highest 11C-methionine uptake was measured in the liver and PTN, followed by the kidneys and the primary tumor. The muscle, blood and plasma DAR values of tumor bearing rats did not differ from those of control rats by using the two radiotracers. Low chronic accumulation that would have indicated infection or spontaneous tumor formation elsewhere was not found.

3.3. Lymphoid spread of metastasis

To decide whether My2/De cells migrate to the PTNs by hematogeneous or lymphoid spread, My2/De cells (10^6/rat) were administered i.v. or i.p. to two groups (4 rats each). It was found that only the i.p. treatment resulted in the enlargement of PTNs in conformity with earlier observations with hepatocarcinoma (HeDe) and nephroblastoma (NeDe) cells that migrated from the disrupted primary tumor of the kidney to the abdominal cavity and through the diaphragm to the thoracic lymph nodes, primarily to the PTNs [27].

To mimic the transport of My2/DE cells, India ink colloidal particles were injected intraperitoneally and their migration was followed. India ink particles appeared 72h after i.p. injection in the liver, spleen and PTNs, but not in the thymus (Fig. 3, boxed area). When India ink was given i.v., these colloidal particles accumulated only in the liver and the
spleen, but not in the thymus or parathymic lymph nodes (not shown), confirming the lymphoid spread of colloidal particles.

3.4. Accumulation of radiotracers in My2/De cells

The in vitro accumulation of $^{18}$FDG, in control and My2/De leukemic cells freshly isolated from the bone marrow of tumor-bearing rats was compared. The radiotracer uptake lasted for 15, 30 or 60 min (Fig. 4A). The basic $^{18}$FDG uptake in control cells after 0, 15, 30 and 60 min increased from 0 to 0.05, 0.08 and 0.17%, respectively. In tumorous rats the $^{18}$FDG uptake was significantly higher: 0, 0.36, 0.77 and 1.3%, respectively. Similar tendency was observed when $^{11}$C-methionine uptake was measured. The 60 min uptake of $^{11}$C-methionine in control bone marrow cells was less than 0.5%, in the bone marrow cells of tumor-bearing rats 4.7% (Fig. 4B).

3.5. Organ enlargement upon subrenal implantation

As an indicator of My2/De cell spread, the enlargement of affected organs (liver, kidney, spleen, PTN) was tested 4 weeks after SRCA implantation. These experiments showed the enlargement of liver, kidney, spleen and PTN upon My1/De implantation (Fig. 5 and Table 2). My2/De cells proliferated to a significantly lesser extent than My1/De cells (not shown), indicating that the two leukemias are different.
3.6. Cytochemical staining

Cytochemical staining was performed in cells obtained from different organs of My2/De leukemic rats. Giemsa dye stained blast cells of the blood (Fig. 6A and B), resting and dividing monoblasts of the bone marrow (Fig. 6C and D). Methanol-Giemsa staining of My2/De spleen count revealing the presence of monocytes in tumor-bearing rats (Fig. 6E and F). May-Grünwald positive staining of monocytes in tumorous PTNs (Fig. 6G and H). Giemsa staining against control (Fig. 7A) was repeated after 24 h (under 400x magnification) (Fig. 7B) and 48 h (800x magnification) cultivation of My2/De cells (Fig. 7C) isolated from bone marrow showing monocytes that turn to macrophages.

3.7. Differential staining of My2/De cells

Myeloperoxidase reaction showed fine brownish staining granularity in the blood count and in the monocytes of My2/De leukemic rats (Fig. 8A and B). Similarly to the myeloperoxidase reaction, Sudan Black B staining distinguishes between acute lymphocytic leukemia and acute myelogenous leukemia. Monocytes and macrophages do not give the reaction except monoblastic/monocytic leukemia cells. The staining of bone marrow monocytes with Sudan Black B indicates the monocytic origin of My2/De cells (Fig. 8C and D). The acid phosphatase reaction confirmed the monocytic origin of My2 leukemia in the bone marrow. Acid phosphatase activity was detected in the 24h bone marrow cell culture (Fig. 8E and F). The β-naphthyl-acetate esterase as the most suitable reaction for the identification of monoblastic types of leukemia served to make distinction between neutrophil and monocyte lineages. As expected the control blood contained fewer large monocytes than the blood of the leukemic blood (Fig. 8G and H).

4. Discussion

Blood, bone marrow, spleen counts, autopsy findings and cytochemical reactions served the characterization My2/De cells. The myeloperoxidase reaction reflected myeloid progenitor positivity in the bone marrow count. The myeloid origin of My2/De cells was documented by the Sudan Black B reaction showing the presence of monoblast-like cells. The monocytic origin of My2/De also evidenced by β-naphthyl-acetate esterase and acid phosphatase reactions was an important criterion to test the metastatic spread of large leukemia cells.

Cells obtained from solid tumors of rats (hepatocarcinoma, nephroblastoma) implanted under the subrenal capsule and migrated to the parathymic lymph nodes [27], raising the possibility of a general metastatic route. To follow the metastatic spread of larger cells, My2/De were subjected to i.v. and i.p. administration and implantation under the renal capsule of rats. Intravenous administration caused the hematogenous spread of My2/De cells without entering PTNs. Subrenal implantation of My2/De cells generated leukemia in abdominal organs and projected lymphatic metastasis to PTNs. Intraperitoneal administration of tumor cells resulted in lymphoid metastasis, manifested by the appearance of My2/De cells in the parathymic lymph nodes. The metastatic spread to PTNs was followed by radiotracers and confirmed by PET analysis.
Similarly to My2/De, My1/De cells are enlarged relatively with a nucleus of 10–15 μm in diameter [19]. Unlike erythrocytes, enlarged leukemia cells are less easily deformed, change the rheology of blood that can hinder passage through the blood capillaries and lead to the interruption of blood flow and tissue hypoxia [28]. The blockade of lymphangiogenesis channeling tumor cells to other lymphatic vessels rather than to blood vessels can be explained by the high permeability of lymphatic capillaries [29]. Instead of vascular uptake, the lymphatic spread of large leukemia cells seems to be a reasonable alternative. Lymphatic vessels of 56.3 ± 9.0 μm capillary diameter can dilated up to nearly 100 μm [30] versus the 5–10 μm diameter of arterial or 7–50 μm venular capillaries.

To mimic the lymphatic spread, the i.v. and i.p. injection and subrenal implantation of India ink colloidal particles provided the key to the metastatic spread of My2/De leukemia. As expected intravenously administered colloidal particles were filtered out and accumulated in the liver and in the spleen but not in the PTNs. Intraperitoneally injected colloidal was taken up by the abdominal organs, primarily by the liver and spleen, and by the PTNs taken through the lymphatic capillaries. In agreement with earlier findings subrenal implanted ink remained in the kidney and appeared in the parathymic lymph nodes [31]. Experiments with India ink led to the conclusion that colloidal particles (tumor cells, India ink) enter the transdiaphragmatic lymphatic channels and drain to the intrathoracic lymphatics [27]. Other authors have also found that beside PTNs i.p. administered India ink appeared in mediastinal lymph nodes [32].

The fact that colloidal particles cross transdiaphragmatic channels and drain to the thoracic lymphatics, enter parathymic, mediastinal, among them anterior mammary lymph nodes could reflect a so far unknown mechanism of metastatic development. This potential connection led to the view that breast cancer development could be a metastasis, rather than a primary tumor [33,34]. When tumor cells leave the blood circulation, e.g. during spontaneous splenic rupture in acute lymphoblastic leukemia, tumor cells are released into the peritoneal cavity, and spread through a lymphatic rather than a regular circulatory route. As far as the pathologic rupture of spleen is concerned it was seen as the initial manifestation of acute myeloid leukemia of unknown mechanism [35]. The occurrence of splenic rupture in acute myeloblastic leukemia is a well known complication of AML [36–47]. A recent systematic review revealed 613 cases of splenic rupture, to highlight the surprisingly frequent occurrence of splenic disruptions [48].

Delayed vascular movement and splenic disruption can release large lymphoblastic leukemia cells into neighboring tissues and body fluids and promote the formation of micrometastases. Animal models did not give definitive answer whether the broken away cells: (i) are engulfed by tissue macrophages, (ii) are drained into the nearest so called sentinel lymph node, (iii) return to the blood stream, or (iv) spread through the interstitial fluid to different locations of the body. Regarding tumor cells released from major peritoneal and retroperitoneal organs (liver, spleen and kidney) and other small particles (India ink, bacteria, nanoparticles) as antigens, further model experiments are expected to reveal the spread and to clarify the route of metastasis and to design alternative solutions for the prevention and treatment of metastatic tumor spread.

![Fig. 6. Giemsa staining of My2/De cells isolated from different organs of control and tumor-bearing rats. The control rats were implanted with a Gelasporn® disc without tumor cells. The other rat was implanted 10² My2/De cells. Rats were euthanized one month after the implantation. (A) Giemsa stained control blood count. (B) My2/De leukemia blood count. (C) Giemsa stained control bone marrow. (D) Bone marrow cells of tumor bearing rat with resting (a) and dividing mononoblasts (b). (E) Methanol-Giemsa stained control spleen count. (F) My2/De spleen count: (a) Red blood cell, (b) lymphocyte, and (c) resting monoblast. (G) May–Grünwald–Giemsa staining of control PTN cells. (H) My2/De cells derived from PTN with small lymphocyte (a), lymphocyte (b), and monoblast cell type (c). Bars, 50 μm each.](Image)

![Fig. 7. Cytochemical reactions of My2/De cells. Blood cells of control and leukemic rats 4 weeks after implantation were stained. Myeloperoxidase reaction in the blood count of control (A) and My2/De rats (B). The reaction is negative in all cells resembling monoblasts. Sudan Black B of control (C) and My2/De cells (D). The large dividing mononoblast-like cell shows brown-like diffuse staining positivity. (E) Acid phosphatase reaction in the control 24 h bone marrow culture with small cells. (F) Acid phosphatase positivity in the monoblastic monocytes of a 24 h leukemia bone marrow culture. The red staining of cytoplasmic granules of large macrophages are clearly visible. The nucleus is not stained. (G) Alpha-naphthyl-acetate negative blood count in control cells. (H) Alpha-naphthyl-acetate positivity of blood count of My2/De leukemic rat.](Image)
Conflict of interest statement

The authors have no conflict of interest to declare.

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Contributors: A.K. has discovered and P.K. has maintained the tumor cells since 1997. E.N. synthesized 11C-methionine, G.T. made experiments related to radiotracer uptake and PET imaging. G.N., B.K. and P.K. performed the experiments. P.K. and G.T. made the experiments related to subrenal transplantation. G.B. provided the intellectual content and wrote the article.

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