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Engraftment of human blood malignancies to the turkey embryo: A robust new *in vivo* model

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ABSTRACT

Xenografting of human blood malignancies to immunodeficient SCID mice is a powerful research tool. We evaluate here whether the immunodeficient turkey embryo can also serve as a xenograft host for human blood malignancies. Human leukemia, lymphoma and myeloma lines engrafted robustly into medullary and extramedullary tissues of turkey embryos as detected by PCR, FACS and histology in 8–10 days. Four of eleven patient AML samples also engrafted the bone marrow. Grafts of two lines responded to chemotherapy with doxorubicin. The turkey embryo therefore has the potential to be a complementary xenograft model for the study of human blood malignancies.

1. Introduction

1.1. Xenograft models for studying human blood malignancies and testing drug efficacy

Immunodeficient mice have been used extensively to study human blood malignancies. For example, a recent review of animal models of AML in immunodeficient mice cited more than 450 articles ([1]). Substantial engraftment of human leukemias in mice takes at least 6 weeks [2], unless cells are grafted directly to the bone marrow (BM) using a technically demanding procedure ([3]). Transplantation of human leukemia to mice has been used to address basic questions in leukemia biology such as identification of leukemia initiating (stem) cells (i.e. [4]) and the molecular basis of engraftment and dissemination ([5]), as well as for clinically-related studies, such as predicting prognosis and relapse of specific patients ([6–8]). Although powerful, the murine model is expensive, requires long engraftment times, and involves increasingly stringent animal experimentation regulations.

1.2. The chick embryo as a host for human cancer

Xenotransplantation of human cells and tissues to the embryonic chick [9] and, particularly, to its chorioallantoic membrane (CAM) is a well established system for evaluating angiogenesis in solid tumor growth (i.e. [10]) and anti-neoplastic drug screening (i.e. [11]). The CAM model reproduces many of the characteristics of tumors *in vivo*, such as tumor mass formation, angiogenesis and metastasis. For example, transplantation to the chick CAM was used to study the dissemination pathway of eGFP-labeled melanoma cells [12]. The chick embryo is naturally immunodeficient, requires no maintenance, and is extremely accessible to experimental manipulation Thus, advantages of the chick embryo over the NOD/SCID mouse models include simplicity, lower cost, and reduced use of sentient laboratory animals.

We recently demonstrated that the human leukemia line K562 engrafted the hematopoietic organs and extramedullary sites in chick embryos ([13]). 100% of embryos engrafted when cells were injected intravascularly (IV) or into the amnion, and the engraftment was detectable at 1–2 weeks. Proof-of-principle for using the chick egg system for chemotherapy testing was also obtained. More recently it was shown, that injections of non-transformed human hematopoietic stem/precursor cells into very early (2 day

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incubation) chick embryos, resulted in engraftment of several loci of leukocyte formation [14].

1.3. Need for an improved system

In our initial studies of the avian egg as a host for human blood malignancies, the level of engraftment of K562 was quite low with intravenous (IV) injection which best simulates the disease process (0.001–0.01% after 10^7 cells were injected). In addition, when two additional laboratory leukemia lines, were injected either IV or into the amnion of chick embryos they engrafted only in the CAM or not at all. Furthermore, eggs injected with patient leukemia cells did not engraft. We therefore sought to improve the model by using the eggs of the turkey (Meleagris Gallopavo) with an incubation period of 28 days to hatching, compared to 21 days for the chicken, to allow more time for engraftment.

1.4. The turkey embryo is much superior to chick embryos as a host for human blood malignancies

We report here that turkey embryos are a much superior host for human blood malignancies than chick embryos. IV injected leukemia lines including K562, Jurkatt, DAMI, G2 and HL-60 engraft at 100% consistency into the bone marrow of turkey embryos as do several human myeloma and lymphoma lines. Engraftment was detected by PCR, flow cytometry and histological methods. Importantly, about 1/3 of patient cell samples injected IV engrafted in the turkey embryo bone marrow. Treatment of leukemia engrafted turkey embryos with doxorubicin dramatically reduced tumor burden within days, indicating that this model can be used for rapid and inexpensive chemotherapy testing. We conclude that turkey embryo has the potential to become a simple, inexpensive and humane model for studying of many aspects of human blood malignancies. In addition, to the best of our knowledge, this is the first use of an avian species other than the chick Gallus Gallus, as a robust host for human cancer cells.

2. Methods

2.1. Cell lines

The following human cell lines were used in the study: leukemias K562, DAMI, Jurkat, HL-60, G2, and CCRF; myelomas CAG and U266, lymphomas Raji and HCL-2 and an EBV-immortalized line of normal human lymphocytes (purchased from the National Laboratory for Genetics of the Sackler School of Medicine of Tel Aviv University). eGFP-expressing sublines of K562, Jurkat, DAMI and CAG were engineered and used in some experiments ([13] and acknowledgements). Lines were maintained for not more than 1–1.5 months after defrosting, using standard conditions. After initial mycoplasma testing of all lines received in the laboratory, pooled samples of all lines are tested weekly/biweekly for mycoplasma contamination (PCR mycoplasma test kit, Biological Industries, Bet-HaEmek, Israel), and no contaminated cells were detected in the course of this study. The CAG myeloma line grew as two populations, adherent and non-adherent [15], and we used only adherent CAG cells for analysis (see Section 3). Blood and bone marrow cells were obtained from surplus material in freshly discarded material up to 24 h after collection. Viability of fresh cells was always >90% and of cryopreserved cells after defrosting >60%. Cytogenetic testing of patient samples was performed by the Cytogenetics Lab of the Department of Genetics, Tel Aviv Medical Center and molecular testing was performed by the Molecular Biology lab of the Department of Bone Marrow Transplants, Tel Aviv Medical Center. This study was approved by the Tel-Aviv Sourasky Medical Center ethics committee according to the Helsinki Accords.

2.2. Injections of blood malignancies to avian embryos

In our previous study of chick embryos, we found that both IV and intra-amniotic injections of K562 leukemia cells resulted in consistent engraftment. We obtained higher levels of human cells with the intra-amniotic injections than with IV injections [13]. However, IV injection is a better delivery mode for dissemination of blood malignancies than amniotic injections and since it is similar to the standard SCID mouse model, we focused our efforts on IV injections.

Fertile turkey (Meleagris Gallopavo) and chicken (Gallus Gallus) eggs were obtained from local farms. For IV injections of blood malignancies to embryos, eggs were incubated with turning at 37.5° for 11–13 days. A window in the shell was cut with surgical scissors, and 1–10 × 10^6 cells derived from lines derived from human blood malignancies or patient cells were injected through the shell membrane into large chorioallantoic membrane (CAM) vessels. The eggs were then sealed with standard office supply cellophane tape, and returned to the incubator for an additional 2–13 days. Embryos were removed on E19–25 (at least 3 days before hatching), and sacrificed by rapid decapitation. The bursa of Fabricius, bone marrow (BM), brain, spleen, liver and the posterior portion of the brain were removed and rapidly frozen for PCR analysis, or fixed with 4% paraformaldehyde or Bouin's fluid for histological analysis, or dissociated and prepared for flow cytometry. In a few experiments, the thymus was analyzed as well. When eGFP-expressing cells were used, some embryos were dissected and the chorioallantoic membrane and internal organs examined macroscopically using a fluorescence-equipped stereomicroscope (Olympus SZX-10). A schematic illustration of the general experimental methods used is shown in Fig. 1.

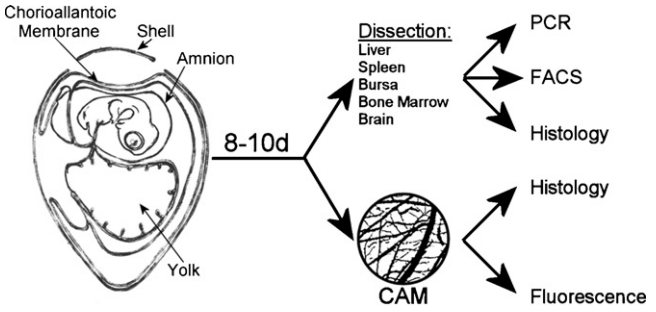

Fig. 1. Schematic representation of IV injection of human blood malignancies into turkey embryos and subsequent analysis. On embryonic day 11 to 13, a window in the egg was made by cutting out a piece of the shell, exposing the chorioallantoic membrane (CAM). Cells were injected into a large CAM vessel, and the egg returned to the incubator for 8–10 days in most experiments. After allowing time for engraftment, the embryo was removed from the egg, and multiple tissues dissected out for PCR, FACS or histological analysis. In parallel, the CAM was spread out intact in a petri dish and examined in a dissecting microscope equipped with epi-fluorescence to view those lines that were engineered to express eGFP. When fluorescence was observed, some portions containing human cells were dissected and prepared for histological examination.

2.3. PCR analysis

DNA was extracted from frozen tissues using standard phenol extraction or using a kit (Promega #A1125). Human DNA was detected using primers to the repeated human-specific alpha-satellite [16] or Alu sequences [17]). The number of cycles was 32–35 for either alpha satellite or Alu primers. Human DNA (positive control) and turkey DNA (negative control) were run with each PCR reaction. When it was necessary to compare groups of experimental embryos, in the case of chicken vs. turkey or chemotherapy-treated vs. controls, the ImageJ program was used to estimate the amount of DNA in PCR bands [18].

Primers:

Alpha-satellite: amplification product—476 bp
GGG ATA ATT TCA GCT GAC TAA ACA G—forward
AAA CGT CCA CTT GCA GAT TCT AG—reverse
Alu: amplification product—238 bp
CAC CTG TAA TCC CAG CAC TTT—forward
CCC AGG CTG GAG TGC AGT—reverse

2.4. Quantitative-PCR

Real-time quantitative PCR was performed using Syber Green dye and alpha-satellite primers. A series of 10-fold dilutions of human leukemia in turkey bone marrow cells were made for each cell line examined, and DNA extracted. Four control dilutions were run in parallel with the experimental samples (100%, 10%, 1% and 0.1%) and the percentage engraftment was computed using Opticon Monitor software (MJ Research).

2.5. Flow cytometry

Turkey embryos were injected IV with 5 × 10^6 K562 cells on E11 and sacrificed on E21. Liver, spleen and BM were analyzed. To obtain single cell suspensions of bone marrow, both femurs were removed and the ends of the bone sliced open. Each bone was flushed with 1 ml of CaMg-free PBS, and single cell suspensions prepared using a 40 μm nylon mesh. The liver and spleens were disassociated by mincing with a scalpel and filtered. Suspensions were incubated with anti-human CD71–FITC antibodies (Dako, Glostrup, Denmark) for 30 min at 4 °C. At least 10,000 events per