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ARTICLE

A WT1 Expressing Metastatic Human Kaposi Sarcoma Xenograft Model

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We have established a non-metastatic and a metastatic human Kaposi sarcoma (KS) xenograft model in SCID mice by injecting KS-Imm cells subcutaneously and intrasplenically, respectively. KS-Imm cells expressed endothelial markers, CD34 and vWF in vivo. Futhermore, we have shown that these cells express all the splice variants of the WT1 gene and WT1^{wt} protein in vitro and in vivo detected by nested PCR and immunohistochemistry. WT1 expression in the peripheral blood was only detectable in case of metastatic KS model suggesting it as a molecular marker of progression. (Pathology Oncology Research Vol 10, No 1, 22–25)

Keywords: Kaposi sarcoma, xenograft model, WT1 expression, metastasis

Introduction

Kaposi's sarcoma, a malignant tumor of endothelial cells, appears in four variants, classical skin, African endemic (skin), post-transplant and AIDS-associated forms, all caused by the HHS-8 virus.[1,2] While the classical form involves the skin, the post-transplant and AIDS-associated forms behave more aggressively able to metastasize to various visceral organs. The KS lesions contain spindle cells, precursor endothelial cells, macrophages and dendritic cells. KS was considered to be derived from endothelial cells based on the expression of CD31, CD34, vWF and c-kit in spindle cells.[3] However, recent studies revealed that KS cells are transformed lymphatic endothelial cells expressing lymphatic markers such as VEGFR3[4,5] and D2-40[6] and the fibroblastic marker 1B10.[7] Bone marrow stem cells (BMSC) are characterized by the constitutive expression of c-kit and the WT1 genes[8-11] and we have postulated that the expression of the latter may be maintained during the differentiation of BMSC to various endothelial cells, including lymphatic endothelia.

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Progression of the post-transplant and AIDS-associated KS is a serious clinical problem which does not have established sensitive diagnostics and effective therapy, therefore an appropriate animal model of human KS could have significance. There are a few xenograft models available for preclinical studies, all but one of which are non-metastatic therefore they have limited experimental usefulness.[12,14]

The purpose of this study was to establish a metastatic human Kaposi sarcoma xenograft in SCID mice and analyze the WT1 expression during its experimental progression.

Material and Methods

Cell line

KS cell line was derived from human Kaposi sarcoma[12] and was cultured in RPMI containing 5% FCS.

Human Kaposi sarcoma xenografts and metastasis models

In vitro cultured KS cells have been injected s.c. into SCID mice with the cell number of $5x10^5$ cells/animal. The tumors became measurable within 7 days and killed the animals within 35-40 days. For experimental liver metastasis assay in vitro cultured KS cells have been injected into the spleen of SCID mice at a cell number of $5x10^5$/animal. Four weeks later animals have been overdosed with Nembutal, the internal organs were removed and analyzed microscopically.

During the in vivo experiments each week blood was drawn from the animals, treated with EDTA and nucleated cells were isolated with Red Blood Cell Lysing Buffer (SIGMA).

Histology and immunohistochemisty

Experimental tissue samples have been embedded into paraffin, sections were cut and stained with H&E. For antigen retrieval paraffin sections were treated with microwave as described.[8] To identify endothelial cells sections were stained regularly for human CD34, CD31 and F-VIII/vW using human specific monoclonal antibodies (DAKO, Glostrup, Denmark). The bound antibodies have been revealed by LSAB2 kit (DAKO) and AEC was used as chromogen. Nuclei were counterstained by hematoxylin.

To detect WT1 protein microwave treated paraffin sections were labeled with mouse monoclonal anti-human WT1 antibodies (Clones: 6F-H2^{wt} and 2C12,WT1^{17AA+} Pharmigen). The bound primary antibody was revealed as above. As positive control, human Wilms tumor tissue samples were used.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from tumor samples and lymphocytes using Trizol reagent according to the instructions of the manufacturer. 1 μg of total RNA was reverse transcribed using oligo(dT)[12-18] primer (Gibco/BRL) and M-MLV reverse trascriptase (Sigma). The reaction mixture was incubated at 37°C for 50 min, heated at 85°C for 20 min, and then stored until use at –20°C.

WT1 nested PCR

DNA amplifications were performed using DyNAzyme^{TM}II (Finnzymes) and Mastercycler gradient thermal cycler supplied by Eppendorf. A master mix of reagents for samples was prepared, containing reaction buffer (10 mM Tris-HCl pH=8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton®X-100), 200 μM/each dNTP, 1,00 pM/reaction each primer, 0.8 U of DyNAzyme^{TM}II polymerase/reaction in the first step and 0.25U DyNAzyme^{TM}II polymerase/reaction in the nested step.

Primers and PCR conditions used for human Wilms' tumour 1 gene (WT1 – NM-024424) were as follows.

P*rimer pair for the KTS region, outer* WTO/1: GGC ATC TGA GAC CAG TGA GAA; WTO/2: GAG AGT CAG ACT TGA AAG CAG T (denaturation: 94°C, 1 min; primer annealing: 64°C, 1 min; chain elongation: 72°C, 2 min – 30 cycles).

Inner primer pair for the KTS region WTI/1: GCT GTC CCA CTT ACA GAT GCA; WTI/2: TCA AAG CGC CAG CTG GAG TTT (denaturation: 94°C, 1 min; primer annealing: 64°C, 1 min; chain elongation: 72°C, 2 min – 35 cycles).

Outer primer pair for the 17AA region WT17aa/1: GCT GCT GAG GAC GCC CTA CAG CAG; WT17aa/2: CAA GAG TCG GGG CTA CTC CAG G (denaturation: 96°C, 95°C, 94°C, 1 min; primer annealing: 65°C, 1 min; chain elongation: 72°C, 1 min – 2+2+36 cycles, touch down).

Inner primer pair for the 17AA region 171/1: CAG ATG AAC TTA GGA GCC ACC; 171/2: CAC ATC CTG AAT GCC TCT GAA (denaturation: 94°C, 1 min; primer annealing: 57°C, 1 min; chain elongation: 72°C, 2 min – 35 cycles).

Human β*-actin primer pair*: βS4: GTG GGG CGC CCC AGG CAC CCA; βA4: GTC CTT AAT GTC ACG CAC GAT TTC (denaturation: 94°C, 1 min; primer annealing: 60°C, 1 min; chain elongation: 72°C, 2 min – 21 cycles).

After amplification 10 μl of PCR products were separated on 4% (KTS region) and 2% (17AA region) agarose gel and stained with ethidium bromide.

Results

Establishment of metastatic human Kaposi sarcoma xenograft

KS cells were tumorigenic when injected s.c. into SCID mice, resulting in large tumors. However, these cells were not metastatic from this orthotopic site and no organ metastases developed even after long-term follow-up of the animals. KS cells formed solid epitheloid tumor cell nests *(Figure 1a)* maintaining endothelial characteristics in vivo, based on the diffuse vWF/F-VIII- *(Figure 1b)* and scattered CD34 expressions *(Figure 1c)* demonstrated by immunohistochemistry. On the contrary to the skin, when KS cells were injected into the spleen, beside the primary tumor *(Figure 2a),* liver metastases have been developed in all animals (5/5) *(Figure 2b).*

Expression of WT1 in KS cells

Expression of WT1 gene was analized by nested PCR of subcutaneous, splenic and liver KS lesions *(Figure 2c).* These studies suggested that KS cells express all KTS+/- and 17AA+/- isoforms of WT1, both in vitro and in vivo. To confirm this conclusion, we have tested the WT1 protein expression in the primary splenic tumors and in their liver metastases by immunohistochemistry as well using two antibodies, 6FH2, a wild-type – and 2C12, a 17AA – specific antibodies. Data revealed that the WT1^{wt}-specific antibody recognizes WT1 protein in the cytoplasm of KS cells exclusively and no nuclear reaction is detectable in these cells *(Figures 2.d,e),* while 2C12 antibody did not detect protein at all (data not shown).

Use of WT1 expression for the follow up of hematogenous dissemination of KS cells in vivo

We have attempted to detect circulating KS cells from the peripheral blood of mice carrying primary spleen KS

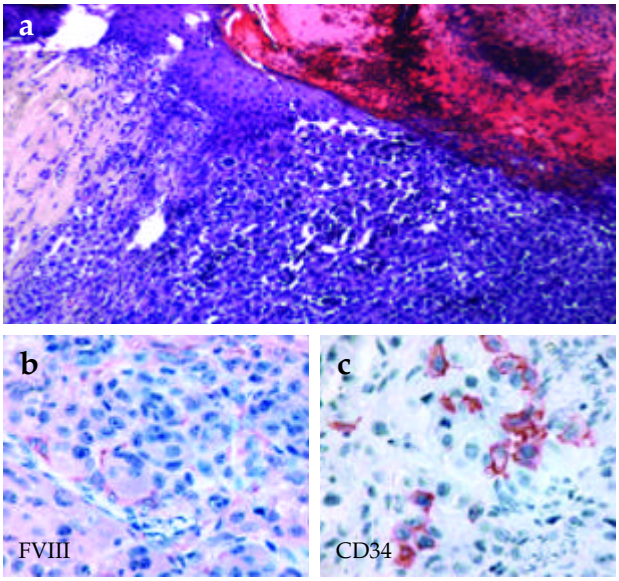


Figure 1. *Histology of KS xenograft in SCID mice following s.c. injection. Morphology of skin primary tumor.* ***(a)*** *Note the ulcerated surface of the skin which is underneath contained solid tumor mass composed of epitheloid tumor cells. (x20) Immunohistochemistry revealed diffuse cytoplasmic positivity for F-VIII antigen in KS cells* ***(b)*** *and strong membrane positivity for CD34 in a smaller proportion of tumor cells* ***(c).*** *(x40)*

tumor. On the 35th day following intrasplenic injection, when liver metastases were already present, WT1 mRNA was detectable in nucleated cells of the peripheral blood in 2 out of 5 animals *(Figure 3a)*. Circulating tumor cells entrapped in the lung have also been detectable by WT1-immunohistochemistry *(Figure 3b)*. The specificity of this finding was confirmed by the analysis of the peripheral blood of animals having nonmetastatic subcutaneous KS tumors. In these animals the peripheral blood was repeatedly negative for WT1 mRNA (3/3, *Figure 3a*).

Discussion

Here we documented the establishment of metastatic human KS xenograft using the KS-Imm cell line and SCID mice. Although KS cells were tumorigenic following transplantation into various rodent tissues (skin or spleen), metastatic potential was developed in the spleen exclusively. This phenomenon is quite interesting, since cutaneous KS forms (classical variant) are relatively benign lesions rarely progressing to other organs. On the other hand, our model suggests that the same KS tumor cell in visceral organ such as the spleen, harbors metastatic potential and this conversion of the model is similar to the post-transplant- or AIDS-associated disease. We suggest the ortho- or heterotopic injection model of human KS-Imm in SCID mice as a relevant preclinical model of human KS.

Recent data suggest that KS cells are derived from endothelial cells but there is a considerable debate if the origin is vascular of lymphatic endothelium. Traditional histopathologic markers of KS are endothelial markers: CD31 and vWF are not sensitive enough while CD34 is not specific for KS cells. Recently VEGFR3 was shown to be a highly selective and specific marker for KS suggesting that this tumor may be derived from lymphatic vessels.[4,5] Our novell finding in this model system of KS was that KS-Imm cells expressed the bone marrow stem cell marker, WT1 gene, in vitro as well as in vivo both at mRNA as well as protein levels. Analysis of the splice variants of WT1 indicated the expression of all the known variants at mRNA level (17aa+/- and KTS+/-) in KS-Imm cells. Although the 17aa+ message was detectable in KS-Imm cells, this protein was not found in KS-Imm xenografts. This might suggest an

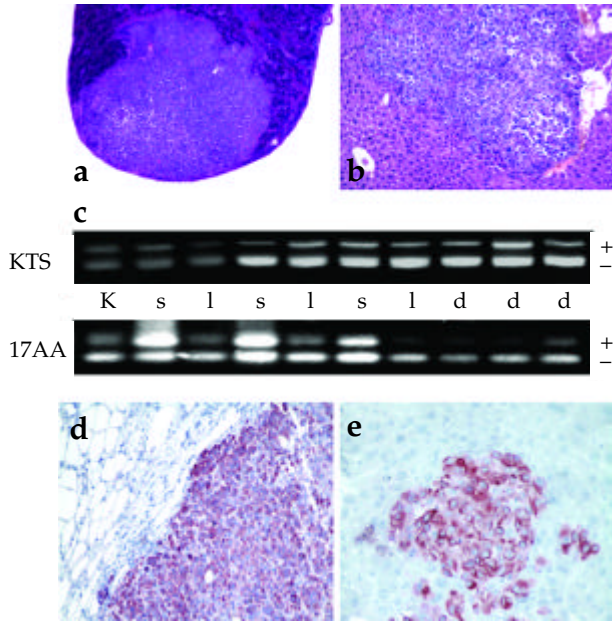
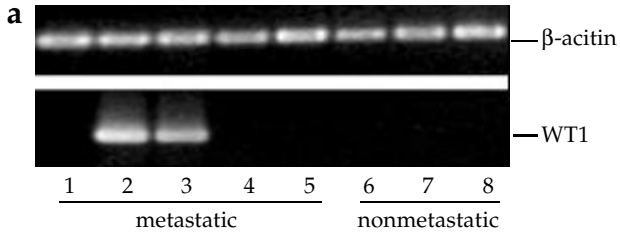


Figure 2. *Expression of WT1 in metastatic KS xenograft in SCID mice.* ***(a)*** *Histology of the the spleen primary KS xenograft (HE, x4),* ***(b)*** *Histology of liver metastasis of KS xenograft on day 35 following i.s. injection (HE, x20),* ***(c)*** *Detection of WT1 splice variants (KTS and 17AA) in KS xenografts by nested PCR. KS= in vitro cultured KS cells, d= s.c. primary tumor, s= spleen primary tumor, l= liver metastasis. Note the balanced expression of KTS+/- isoforms in all the tissues studied compared to the predominant expression of the 17AA- isoform in the skin tumors and liver metastases. Spleen primaries expresses predominantly the 17AA+ isoform of WT1.* ***(d)*** *Detection of WT1 protein in the spleen primary of KS cells detected by immunohistochemistry using 6FH2 antibody. Note the strong cytoplasmic red signal in the cytoplasm of all tumor cells.* ***(e)*** *Detection of WT1 protein in the liver metastasis of KS cells detected by immunohistochemistry using 6FH2 antibody. Note the strong cytoplasmic red signal in the cytoplasm of all tumor cells.*



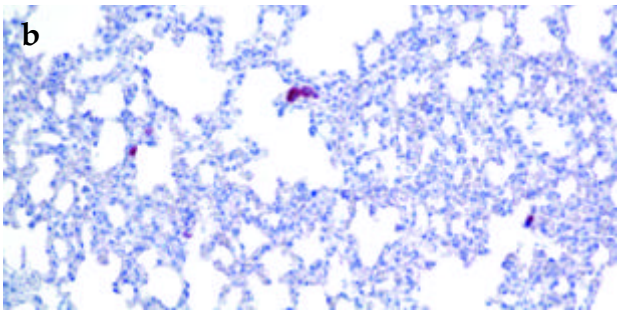


Figure 3. *Detection of human WT1 gene expression in the peripheral blood and lung of SCID mice 35 day following i.s. or s.c. injection of KS cells.* ***(a)*** *WT1 expression as detected by nested PCR. 1-5: Peripheral blood of 5 animals following i.s. injection, lanes 6-8: peripheral blood of 3 animals following s.c. injection. Note that 2 out of 5 animals with spleen primary tumor KTS- isoform of WT1 was detectable (lanes 2,3).* ***(b)*** *Immunohistochemistry of WT1 in the lung. Lung tissue of SCID mice was fixed, embedded into paraffin and sections were labelled for* ^{wt}*WT1 as in case of* ***Figures 2d,e.*** *Note the single KS-Imm cells in the alveolar walls.*

aberrant function of WT1 gene in KS cells. This was further corroborated by the finding that ^{wt}WT1 protein was found in the cytoplasm of KS-Imm cells but not in the nuclei.

WT1 is constitutively active in bone marrow stem cells but is downregulated rapidly parallel to the differentiation toward various hematopoietic cell linages.[8,16,17] On the other hand, the majority of acute myeloid and lymphoid leukemias express WT1 which can be used for monitoring of the disease.[18] We have shown here, that the WT1 expression of KS-Imm cells can be effectively used for monitoring of the progression of the disease. The potential progression marker role of WT1 in this model is supported by the fact that WT1 positivity in the peripheral blood can only be detected when KS-Imm cells formed metastases (following intrasplenic injection) and is not detectable in the presence of cutaneous tumors. Studies on human lesions of various KS forms could test the diagnostic usefullness of this finding.

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