Stem Cells Increase in Numbers in Perinecrotic Areas in Human Renal Cancer

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Abstract

Purpose: Developing strategies to overcome resistance to sunitinib is a major challenge in human renal cell carcinoma (RCC). We hypothesized that sunitinib-induced tumor necrosis–associated hypoxia could interact with renal cancer stem cells in patients with metastatic RCC.

Experimental Design: We studied tissue samples from 7 patients with primary metastatic RCC, before and after sunitinib treatment, and from six xenograft models derived from human RCC. Two xenograft models were responders to sunitinib, the four others were nonresponders. CD133/CXCR4 coexpressing cells derived from the two responder xenograft models were used for in vitro studies.

Results: In the seven primary RCCs, we identified a significantly larger number of CD133/CXCR4–coexpressing cells in perinecrotic versus perivascular areas. Their numbers also significantly increased after treatment, in perinecrotic areas. We reproduced these clinical and pathologic results in all six RCC xenograft models with again a preferential perinecrotic distribution of CD133-expressing cells. Necrosis occurred at day 7 in the two responder models treated with sunitinib, whereas it occurred at day 21 in the untreated controls and in the four nonresponder models. Strikingly, when we studied the six RCC xenograft models at the time necrosis, whether spontaneous or sunitinib-induced, occurred, necrosis area correlated with stem-cell number in all 12 xenografted RCCs. When studied under experimental hypoxia, the number of CD133/CXCR4–coexpressing cells and their tumorigenic potency increased whereas their sensitivity to sunitinib decreased.

Conclusions: In human RCC, sunitinib was able to generate resistance to its own therapeutic effect via induced hypoxia in perinecrotic areas where cancer stem cells were found in increased numbers. Clin Cancer Res; 21(4): 916–24. ©2014 AACR.

Introduction

Antiangiogenic therapies have improved the prognosis of patients with metastatic renal cell carcinoma (RCC), but secondary resistance constantly occurs (1–3). These innovative therapies act through hypoxia-induced tumor necrosis (4), necrosis being considered an indirect marker of oxygen depletion (5).

Preclinical data suggest a central role of hypoxia in the resistance to tyrosine kinase inhibitors such as sunitinib (6, 7). Hypoxia induces resistance to chemotherapy in human cancer progenitors (8, 9), and to targeted therapies in chronic myeloid leukemia cells (10).

Hypoxia and necrosis occur in the spontaneous evolution of cancer. Hypoxia-inducible factor-1α (HIF1α) stimulates angiogenesis by target gene activation (11), and promotes endothelial cell progenitor and cancer stem-cell development (6, 12–14). In tissues, in which microenvironment conditions are thought to govern cancer stem cells, little is known about the role of hypoxia.

According to consensus criteria, cancer stem cells are able to self-renew and have tumorigenic potential (15). Different surface markers characterize them, including CD133, expressed by hematopoietic stem cells (16), renal progenitors (17), brain (18), and colon cancer stem cells (19), and CXCR4, expressed by human hematopoietic progenitors, renal progenitors (20), and pancreatic cancer stem cells (21, 22).

Using CD133 and CXCR4 on human primary RCC biopsies and xenografted samples, we identified stem cells around areas of spontaneous or sunitinib-induced necrosis. When these cells were sorted from xenografted human RCC before treatment, we demonstrated that hypoxia increased their tumorigenic potency, and decreased their sensitivity to sunitinib. Our results, therefore, strongly suggest that in human RCC, sunitinib was able to generate resistance to its own therapeutic effect via induced hypoxia in perinecrotic areas where cancer stem cells are found in increased numbers.

Materials and Methods

Patients and RCC samples

Samples were obtained from primary tumors of 7 patients with metastatic RCC, both before any medical treatment and after 3
months of sunitinib treatment (Pfizer) at 50 mg/d with a 4-week on and 2-week off schedule (1), sunitinib being the first line of treatment. Imaging-guided pretreatment biopsies were similarly performed at a minimum distance of 1 cm from necrotic areas detected on computed tomography, and processed as described in Supplementary Methods. Table 1 shows tumor characteristics.

In compliance with French Bioethics law (2004-800; June 8, 2004), all patients had been informed of the research use of their samples remaining after diagnosis had been established, and did not oppose it. Informed consent was obtained for each patient. The study was approved by the University Board Ethics Committee.

Human RCC xenografts

Six xenograft models of human RCC in nude mice were studied (see Supplementary Methods). The characteristics of the six xenografted human RCC models are shown in Table 2.

The day when tumors reached a volume of 300 mm3—that is, 100% tumor volume—was considered as day 0. Then, 15 of the 35 mice xenografted with human RCC were treated by gavage with sunitinib diluted in 0.9% NaCl, at 20 mg/kg/d.

At day 21, when mice treated with sunitinib were compared with untreated mice, the xenograft model was considered as “responder” to sunitinib if tumor volumes were significantly smaller (P < 0.01).

Table 1. Primary tumor characteristics

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Fuhrman grade</th>
<th>VHL status</th>
<th>Initial TNM statusa</th>
<th>Type of metastasis</th>
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</thead>
<tbody>
<tr>
<td>RCC1</td>
<td>3</td>
<td>Mutated</td>
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<td>pT4NXM1</td>
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<td>Lung, brain</td>
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</tbody>
</table>


Perinecrotic CD133-positive cell counts
We counted CD133-positive cells on tumor samples of primary human RCCs and all RCC xenograft models (see Supplementary Methods).

Assessment of CD133, CXCR4, and HIF1α in perinecrotic cells
For human primary RCCs before and after treatment with sunitinib, multiple-fluorescent immunostainings were performed (see Supplementary Methods).

Assessment of necrosis area
Necrosis areas were assessed on tumor samples from all RCC xenograft models, at days 0, 7, 14, and 21 (see Supplementary Methods).

Cancer stem cells from xenografted human RCC: spheres and tumorigenicity
We studied stem cells from all RCC xenograft models. In the responder and nonresponder models, we analyzed the samples obtained at day 0, before any treatment. Cells obtained from dissociated sections were cultured as described in Supplementary Methods, to obtain spheres. We then assessed the tumorigenicity of CD133/CXCR4–coexpressing cells isolated from these spheres (see Supplementary Methods).

Experimental hypoxia, assessment of proliferation, and microvessel density
We studied spheres obtained from untreated tumor samples (day 0) from the six models. These spheres were separated into two groups, maintained in a humidified chamber for 7 days, one under experimental hypoxia (1% O2) and the other under normoxia (20% O2). Assessment of proliferation and microvessel density was performed on tumor samples of xenografts obtained after engraftment of spheres cultured under normoxic or hypoxic conditions (see Supplementary Methods).

Experimental hypoxia, tumorigenicity, and cancer stem-cell sensitivity to sunitinib
As described in Supplementary Methods, we assessed the relative number of CD133, CXCR4, and CD133/CXCR4–expressing cells in hypoxic and normoxic spheres by flow cytometry. We then assessed tumorigenicity of normoxic and hypoxic spheres, and checked whether CD133/CXCR4 cells from normoxic and hypoxic spheres had different sensitivity to sunitinib.

Statistical analyses
Calculations were carried out using the SPSS Statistics 17.0 software or the R 2.15.2 statistical software (R Foundation for Statistical Computing; see Supplementary Methods).
Results
Stem cells in perinecrotic areas
In the primary tumor samples of the 7 patients with metastatic RCC, before treatment and after 3 months of sunitinib, we identified large cells expressing CD133. When counted independently from their location in the tumor, there was no significant change after treatment (from 2.4% to 3.7%, $P = 0.1$). Interestingly, CD133-expressing cells were significantly more numerous in perinecrotic than in perivascular areas, in both untreated patients (4.7% vs. 1.3%, $P < 0.01$) and treated patients (8.3% vs. 1.9%, $P < 0.01$). In treated patients, CD133-expressing cells were significantly more numerous than in untreated patients but only in perinecrotic areas (8.3% vs. 4.7%, $P < 0.05$; Fig. 1A).

In perinecrotic areas in all patients, 84.5% ($\pm 2.5\%$) of these cells coexpressed CD133 and CXCR4, suggesting a stem-cell phenotype. These CD133/CXCR4-coexpressing cells were significantly more numerous in treated patients compared with untreated patients (7.3% vs. 3.9%, $P < 0.05$). In addition, HIF1$\alpha$, a marker of hypoxia, was coexpressed in 95% of CD133/CXCR4 cells (Fig. 1B).

Six xenograft models of human RCC in nude mice were studied, obtained from engraftment of RCC primary tumor samples from patients with metastatic RCC. Each of the six xenograft models was treated with sunitinib for 21 days. A model was considered as "responder" to sunitinib treatment if its tumor volume at day 21 was significantly smaller than the mean tumor volume among untreated mice ($P < 0.01$). When there was no significant difference in tumor volume between treated and untreated mice, the xenograft model was considered as nonresponder to sunitinib treatment. Two of the six xenograft models were responder to sunitinib, and the 4 others were nonresponder models. In the two xenograft models responder to sunitinib (HRCC1 and HRCC8), a significant increase in necrotic areas was found between days 0 and 7 of treatment with sunitinib (5.4% $\pm 4.1\%$ vs. 32.2% $\pm 10.6\%$ of total tumor surface area for pooled results of these two models, $P < 0.05$). In contrast, it took 21 days to observe necrotic areas both in the group of untreated mice (26.3% $\pm 9.8\%$), and in the group of treated mice not responding to sunitinib (24.9% $\pm 9.4\%$; Fig. 2B and Supplementary Fig. S1).

When we studied the six xenograft models, we again demonstrated that CD133-expressing cells were significantly more numerous in perinecrotic than in perivascular areas, in both treated and untreated tumors. In addition, when we compared untreated and treated mice, CD133-expressing cells were significantly more numerous in treated mice, but only in perinecrotic areas ($P < 0.05$; Fig. 2C and Supplementary Fig. S2).

These six preclinical models enabled us to perform in situ analyses on whole xenografted tumors. After removal of the whole tumor and realization of five representative full sections, the percentages of necrotic areas and of CD133-positive cells were assessed in the five different sections for each tumor (Fig. 2D). When we studied untreated and treated mice together, including responders and nonresponders to sunitinib ($n = 120$ mice), the necrosis ratio in tumor tissue sections was significantly related to the mean number of CD133-expressing cells ($P < 0.01$; Fig. 2D).

When we used pimonidazole, another hypoxic marker, we showed that hypoxic living cells stained for pimonidazole were distributed around necrotic areas. This was observed in the RCC xenograft models when necrosis occurred, at day 21 for one untreated RCC xenograft model, and at day 7 under treatment for one xenograft model that was responder to sunitinib (Supplementary Fig. S3).

Because tissue necrosis is directly related to hypoxia, these results strongly suggest a link between hypoxia, whether sunitinib-induced or spontaneous, and an increased number of RCC stem cells.

CD133/CXCR4 stem cells had tumorigenic potential
To determine whether CD133/CXCR4-coexpressing cells were tumorigenic in vivo, we grew spheres from untreated RCC xenografts. When these spheres were cultured in normoxic conditions, confocal microscopy identified CD133/CXCR4–coexpressing cells (Fig. 3A).

These cells isolated from normoxic spheres induced tumor growth 3 weeks after injection of $2 \times 10^5$ cells into nude mice. This tumorigenic potential was observed with CD133/CXCR4–coexpressing cells from normoxic spheres derived both from the two sunitinib responder models (Fig. 3B) and the four nonresponder models (Fig. 4A).

As controls, $2 \times 10^5$ cells from the CD133/CXCR4 double-negative fraction of normoxic spheres injected in nude mice did not induce any tumor growth over a period of 2 months (Supplementary Table). Microscope analyses and cell counts showed that tumors that developed from CD133/CXCR4–coexpressing cells reproduced the initial primary human RCC features for morphology, cell proliferation, microvessel density, and stem-cell density (Figs. 3B and 4A).

Experimental hypoxia increased stem-cell tumorigenicity
We compared the tumorigenic potential of spheres cultured under hypoxic and normoxic conditions. After injection of CD133/CXCR4–coexpressing cells sorted from spheres, there

<table>
<thead>
<tr>
<th>Tumor model</th>
<th>Pretreatment biopsy</th>
<th>Posttreatment surgery</th>
<th>Fuhrman grade</th>
<th>VHL status</th>
<th>Initial TNM$^*$ status at time of graft</th>
<th>Metastases</th>
<th>Responder to sunitinib</th>
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<td>HRCC1</td>
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<td>pTibM1</td>
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</tr>
</tbody>
</table>

was a significantly larger mean tumor volume after injection of hypoxic stem cells rather than normoxic stem cells \( (P < 0.05; \text{Figs. 3C and 4B}) \). This difference was significant from 42 days on for the two responder models (Fig. 3C) and for the four nonresponder models (Fig. 4B). To rule out a difference due to proliferation or angiogenesis, we counted Ki67- and CD31-expressing cells in tumor sections, and no significant difference was found in tumors grown from normoxic and hypoxic spheres (Fig. 3C).

When we tested the tumorigenic potential of serial dilutions of isolated CD133/CXCR4 cells from normoxic and hypoxic spheres, a significant increase in tumor growth occurred after injection of \( 2 \times 10^2 \) and \( 2 \times 10^3 \) cells when hypoxic spheres were compared with normoxic spheres. The difference was not significant after injection of a larger number of cells \( (2 \times 10^4 \text{ cells}; \text{Figs. 3C and 4C}) \).

**Experimental hypoxia increased the number of stem cells and decreased their sensitivity to sunitinib**

To assess the role of experimental hypoxia on the number of renal cancer stem cells, spheres derived from untreated tumors of the six human RCC xenograft models were cultured under hypoxic conditions. After 5 days of experimental hypoxia, FACS analyses showed a significantly larger number of CD133/CXCR4 cells in hypoxic spheres compared with normoxic spheres \( (P < 0.01) \). This was found in the two responder models \( (7.6\% \pm 2\% \text{ vs. } 2.1\% \pm 0.7\%; P < 0.01; \text{Fig. 3D}) \), and the four nonresponder models \( (9.2\% \pm 2.2\% \text{ vs. } 4.6\% \pm 1.1\%; P < 0.01; \text{Fig. 4C}) \).

To assess sunitinib effects on RCC stem cells, we then analyzed whether CD133/CXCR4 cells from hypoxic spheres were more sunitinib resistant than CD133/CXCR4 cells from normoxic spheres, using an *in vitro* cytotoxicity assay. The IC$_{50}$ value of
sunitinib was higher for hypoxic than for normoxic cells for both the two responder models (Fig. 3D), and the four nonresponder models (HRCC9 to HRCC12).

Discussion

In patients with metastatic RCC who were responders to sunitinib, we identified CD133/CXCR4–coexpressing stem cells in perinecrotic areas, and showed that their numbers increased after sunitinib administration. To our knowledge, this has never been reported in human RCC.

We chose CD133 and CXCR4 because they are two well-known markers of stem cells (16–20). In a series of 240 RCC, more than 20% positive cells were found for CD133-expressing cells in 22.5% of RCC, and for CXCR4-expressing cells in 60.8% of RCC (23), and high CXCR4 expression was predictive of poor response to sunitinib (24). The 7 patients we studied had at least a stable disease under sunitinib treatment (for this reason they underwent nephrectomy after treatment), and when we assessed the number of CD133/CXCR4–coexpressing cells, we found a mean percentage from 4.2% to 7.7%.

To further study these stem cells, we xenografted human RCC in nude mice, and performed a sequential study in untreated and sunitinib-treated mice.
Figure 3.
In the two responder models, hypoxia increased the number of stem cells, their tumorigenicity and resistance to sunitinib. A, under normoxic conditions, confocal microscopy (Zeiss LSM510) shows that RCC sphere cells coexpress the stem cell markers CD133 and CXCR4. B, when grafted subcutaneously, CD133/CXCR4-coexpressing cells isolated by magnetic sorting from HRCC1 or HRCC8 spheres induce tumor growth in two groups of 5 nude mice. At day 63, microscope analyses show that tumors developed from CD133/CXCR4-coexpressing cells reproduce the initial RCC features in terms of proliferation (Ki67-positive cell count), microvessel density (CD31-positive cell count), and stem-cell density (CD133/CXCR4 cell count). C, tumor growth following s.c. injection of 2.10^3 CD133/CXCR4 cells obtained from HRCC1 or HRCC8 spheres cultured either under hypoxic or under normoxic conditions is significantly different (permutation test, P < 10^-5). At day 49, tumor growth for hypoxic cells is larger than for normoxic cells (n = 10 mice in each group); **, P < 0.01. Microscope analyses show that the differences in growth observed between xenografted tumors developed from hypoxic and normoxic spheres do not result from differences between numbers of Ki67- and CD31-positive cells. Follow-up of tumor growth after s.c. injection of serial dilutions of CD133^-/CXCR4^- cells isolated from hypoxic and normoxic spheres shows that only hypoxic cells are able to induce tumor growth at the minimal dilution of 2.10^2 cells, and that they induce a significantly larger tumor growth than normoxic cells at the dilution of 2.10^3 cells (n = 5 mice in each group); *, P < 0.05; **, P < 0.01. MTT assays show that double-positive CD133/CXCR4 cells sorted from hypoxic spheres are more resistant to sunitinib than CD133/CXCR4 cells sorted from normoxic spheres in the two responder models; **, P < 0.01.
increased the number of CD133/CXCR4–coexpressing stem cells with a preferential perinecrotic distribution. When all xenografted human RCC, whether treated or untreated, responders or non-responders to sunitinib, were considered (i.e., 120 tumor samples), we demonstrated that the number of CD133/CXCR4–coexpressing cells was related to the extent of tissue necrosis. In a preclinical model of breast cancer cell lines (7), the number of cancer stem cells increased under sunitinib via hypoxia. Here, we studied renal and not breast cancer, and human tumor samples and not cancer cell lines.

Regarding renal cancer stem cells, CD105, more than CD133, has been recommended following studies using cells selected by flow cytometry (25). However, CD105 is not appropriate for studies in whole-tumor tissue sections, because it is also expressed by tumor endothelial cells (26) and carcinoma-associated fibroblasts (27).

In our preclinical model of human RCC xenografts, the number of stem cells, preferentially distributed in perinecrotic areas, was related to the extent of tissue necrosis, whatever the origin of necrosis, spontaneous or treatment induced.
Hypoxia and Cancer Stem Cells in Renal Cancer

VHL gene mutations are found in 70% of human RCC (28), as was the case in our patients and the xenografted RCC. When present, VHL gene mutations lead to HIF accumulation (29), including HIF\(1\alpha\), recently characterized as a tumor suppressor (30, 31), and HIF2\(\alpha\), characterized as an oncoprotein (30). Because HIFs and necrosis are two indirect markers of oxygen depletion (5), for this study based on human cancer tissue analysis we chose to use the extent of necrosis rather than the number of HIF1\(\alpha\)- or HIF2\(\alpha\)-expressing cells as markers of hypoxia.

After sorting from untreated xenografted human RCC, the CXCR4/CD133 coexpressing stem cells were able to form spheres and to induce tumors in mice, two characteristic features of cancer stem cells (15). We showed in vitro that experimental hypoxia increased both their number and their tumorigenicity. Hypoxia can increase the number of glioblastoma (12) and breast cancer (14, 22) stem cells, but we have demonstrated here for the first time that hypoxia can increase tumorigenic potential and resistance to sunitinib of cancer stem cells from untreated human RCC.

The question of additional hypoxia on RCC-bearing VHL mutation opens discussion of the relative importance of intrinsic and extrinsic hypoxia in RCC cells. It has recently been shown that clear-cell RCC can express low levels of HIF\(1\alpha\), particularly when chromosome 1\(\alpha\q\) is deleted (30, 32). Therefore, it is possible that not all RCC cells have the same constitutive pseudohypoxic level. In our preclinical models, human xenografted RCCs were treated by sunitinib, which affects endothelial cells and induces necrosis (4), and thus hypoxia in tumor cells through an extrinsic mechanism.

In patients with metastatic RCC, resistance to antiangiogenic treatment is a major cause of poor survival (2, 3). In preclinical studies, it has been shown that resistance to sunitinib occurred because cancer cells acquired metastatic potential (33, 34), and that sunitinib mainly acts by vascular effect and induction of necrosis (4). Our demonstration that hypoxia increases the number of cancer stem cells while decreasing their sensitivity to sunitinib implies that the drug, by way of its initial effect on the tumor microvessels, is able to induce mechanisms of resistance to its own effect. This is coherent with the clinical observation showing that the benefit of sunitinib is only transitory (2).

Designing new strategies to optimize antiangiogenic therapies need to take into account the deleterious effect of therapy-induced hypoxia, which, as shown in this study, is responsible for the emergence of cancer stem cells in RCC. Targeting cancer stem cells might, thus, be a promising future research approach to prevent acquired resistance to sunitinib. Molecules targeting endothelial cell progenitors such as TRC105, an antiendoglin antibody, are under development in metastatic RCC (35), but the effect of therapeutic antibodies targeting CXCR4 and/or CD133 on RCC cancer stem cells remains unknown.

The clinical relevance of our results is a major strength of this work, as resistance to antiangiogenic drugs is challenging daily practice in oncology. In addition, this study is to our knowledge the first to be performed on human RCC samples before and after treatment, the relevant results being reproduced in xenograft models responding to sunitinib treatment, thus generalizing the conclusions obtained in the patient samples.

Important data are added to existing knowledge about cancer treatment with antiangiogenic drugs because it opens a new field of pharmacologic research based on the fact that an antiangiogenic drug, initially efficient on a metastatic cancer, is also able to induce resistance to its own therapeutic effect.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M. Varna, S. Germain, A. Janin, G. Bousquet

Development of methodology: A. Janin, G. Bousquet

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Varna, G. Gapihan, P. Ratajczak, C. Leboeuf, N. Setterblad, A. Duval, A. Janin, G. Bousquet

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.-P. Feugeas, C. Leboeuf, G. Bousquet

Writing, review, and/or revision of the manuscript: M. Varna, J. Verine, P. Mongiat-Artus, A. Janin, G. Bousquet

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Varna, S. Tan, I. Ferreira

Study supervision: P. Mongiat-Artus, G. Bousquet

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