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LETTER TO THE EDITOR

Human glioblastoma cells exposed to long-term hypoxia and nutrient starvation stimulated induction of secondary T-cell leukemia in mice

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Secondary leukemia occurs long after remission of the primary tumor with a history of chemo and radiation therapy; however, the mechanism of secondary leukemogenesis is not fully understood.[1] In this study, we demonstrate cases of secondary T-cell leukemia in mice after complete regression of xenograft primary glioblastoma independent of chemo and radiotherapy. Recently, antiangiogenic therapy, often in combination with conventional chemotherapy, has been clinically validated for solid tumors.[2] Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are major regulators of angiogenesis;[3] indeed, a monoclonal anti-VEGF antibody (bevacizumab) and small molecular weight inhibitors of VEGFRs (sorafenib and sunitinib) have improved the therapeutic indexes of advanced malignancies, including the increase in disease-free survival in breast cancer, colorectal cancer, non-small cell lung carcinoma, renal cell carcinoma, hepatocellular carcinoma and glioblastoma. However, antitumor effect of anti-VEGF antibody and VEGF receptor inhibitors concomitant with increased invasiveness, and distant metastasis have been reported using preclinical cancer models.[4] In addition, high-grade glioma patients following treatments with anti-VEGF antibody in combination with irinotecan demonstrated an effective response by the primary tumor, but also a high rate of distant tumor progression including tumor infiltration and vascular co-option.[5] We previously reported that long-term hypoxia and nutrient starvation double-deprivation cycles increased cellular migration, invasion and distant metastasis in a murine cancer model, suggesting that this extreme tumor microenvironment could be a mechanism of the tumor aggressiveness following antiangiogenic therapy.[6]

Glioblastoma is the most common brain tumor in adults and is often rapidly fatal with median survival of less than a year from diagnosis.[7] In contrast, brain tumors in fetal to young individuals may achieve complete regression of the primary tumor with surgical resection along with standard chemotherapy and/or radiotherapy. A major clinical problem of brain tumors in fetal and young individuals can be recurrence of the primary tumor or development of secondary leukemia years after remission of the primary tumor. We hypothesized that non-tumorigenic human glioblastoma (T98G) cells exposed to hypoxia and nutrient starvation may become aggressive and also affect host cells, causing leukemia.

On this basis, we developed a cell culture system to maintain cells under hypoxia and nutrition starvation double deprivation-stress (DDS) cycles, which at least partially reflected *in vitro* the hypoxic and nutrient-starved tumor microenvironment under antiangiogenesis therapy.[6] Using this system, we generated T98G-DDS10 cells exposed to 10 or more cycles of hypoxia and nutrient deprivation stress cycles (Figure 1a), because most cancer cells including T98G cells cannot be maintained under the prolonged hypoxia and nutrient starvation DDS for longer than 72 h *in vitro* (Figure 1b).

To examine whether hypoxia and nutrient starvation facilitate the induction of tumorigenicity in non-tumorigenic T98G cells, 1×10^7 of T98G-DDS10 cells were subcutaneously inoculated into BALB/c nu/nu nude mice ($n = 5$) along with the original T98G cell xenograft in control mice ($n = 5$). T98G-DDS10 tumor xenograft mice demonstrated initial formation of tumors (3/5), but not original T98G tumor xenograft mice (0/5) (Figure 1c). The T98G-DDS10 primary tumor showed complete regression within 2 weeks. We examined whether T98G-DDS10 xenograft cells induced recurrence of the tumor or affected host cells to induce leukemia after remission of the primary tumor. T98G-DDS10 xenograft mice were disease free for 1 year, but suddenly, around day 365–375 after inoculation of T98G-DDS10, these three mice almost simultaneously lapsed into a cachexic condition with an enlarged spleen, liver, lymph node and invasion of immature lymphocytes in hemorrhagic ascites of the abdominal cavity (Figure 1d, Supplementary Figure 1). Quantitative real-time PCR was performed using human-specific primers against h*β-actin* and human glial fibrillary acidic protein (h*GFAP*) for the detection of T98G glioblastoma cells. In addition, mouse-specific primers against T-cell pan-marker m*CD3* and mouse B-cell pan-marker m*CD19* were used for the detection of mouse-derived secondary leukemia in the spleen and liver tissue specimens of T98G-DDS10 xenograft mice (Supplementary Table 1). As shown in Figure 2a, significant upregulation of m*CD3* mRNA levels was observed, but h*β-actin* and h*GFAP* mRNA was not detected in the spleen or liver tissues of mice with T98G-DDS10 xenograft. The mRNA of m*CD19*, a B-cell marker, was found at similar levels in the spleens of T98G and T98G-DDS10 xenograft mice, and was markedly low in the livers of these mice compared with normal spleens (positive controls), suggesting that m*CD19* expression in leukemic cells is negative at the mRNA level (Figure 2a). Histological examination by hematoxylin-eosin (HE) staining showed massive invasion of leukemic cells associated with scattered mitotic cells into the liver and spleen (Figure 2b). Immunohistochemical staining using anti-mCD3 and hGFAP antibodies was performed in 4% paraformaldehyde-fixed spleen and liver specimens of T98G-DDS10 xenograft mice containing a tumor compared with the spleen and liver of the original T98G xenograft mice. Immunohistochemical staining revealed that the leukemic cells observed in the spleen and liver of T98G-DDS10 xenograft mice were CD3-positive, but GFAP-negative (Figure 2b), suggesting that the tumor had not relapsed with human glioblastoma cells. We suggest that non-tumorigenic T98G human glioblastoma cells become aggressive and stimulate the induction of T-cell leukemia following long-term hypoxia and nutrient starvation DDS.

Amplification or insertion of a human oncogene can result in the development of secondary leukemia. To examine whether oncogenes derived from T98G-DDS10 cells were integrated into the leukemic cells, PCR amplification of the human *Alu*-repetitive sequence, which is thought to be co-integrated with ascertain human genes, was conducted using a spleen with leukemic cells of T98G-DDS10 xenograft cachexia mice and

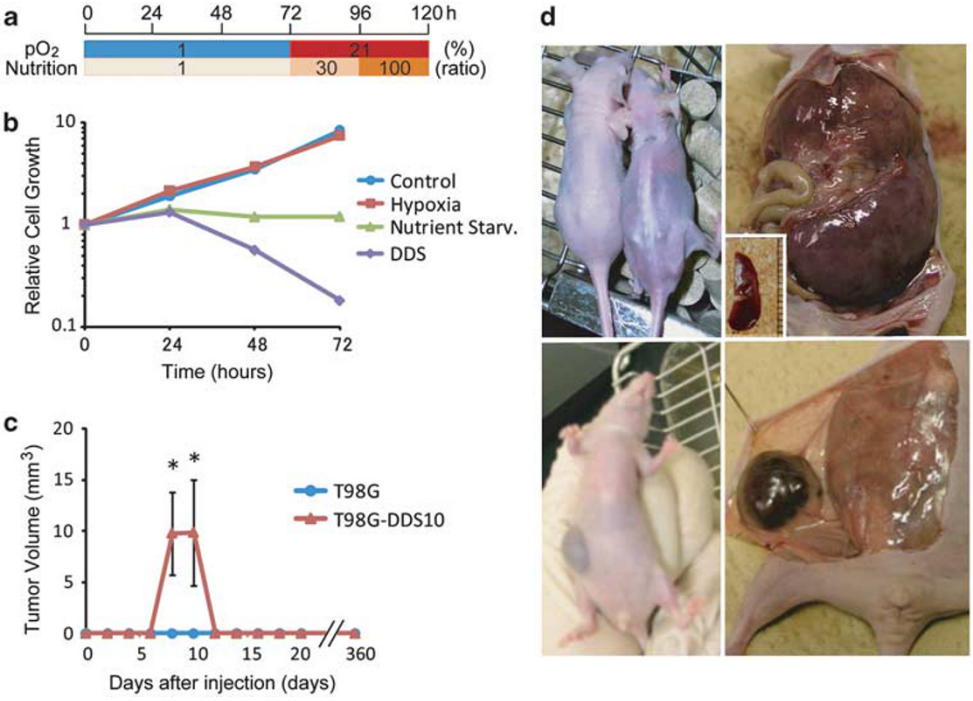

Figure 1 Recurrence of secondary tumor after remission of primary tumor in mice inoculated with hypoxic and nutrient-starved human glioblastoma (T98G) cells. **(a)** Schematic diagram of hypoxia and nutrient starvation DDS cycle. T98G cells were cultured under hypoxia (1% pO_2) and nutrient starvation (nutrient deprivation medium containing 1% of nutrition according to composition of DMEM supplemented with 0.1% fetal bovine serum) for 72 h and recovered by culturing under normoxia in 30% nutrition for overnight, and then 100% nutrition for 24 h until 80–90% confluence was achieved. This hypoxia and nutrient-starvation cycle was conducted for 10 or more cycles. **(b)** Hypoxia-induced cell death under nutrient starvation. Relative cell growth of T98G cells was measured by sulforhodamine B (SRB) cell proliferation assay under the following conditions: normoxia (control), hypoxia (1% pO_2) supplemented with full nutrition (100% nutrition), nutrient starvation (1% nutrition) and hypoxia and nutrient starvation DDS for 72 h. (**c**) Volumes of primary tumor of T98G and T98G-DDS10 subcutaneously injected in BALB/c nu/nu nude mice ($n=5$). (*$P<0.05$, calculated using Student's *t*-test). (**d**) *In situ* pictures of spleen and lymph nodes (blood from abdominal cavity) in mice show secondary tumor.

compared with the spleen of original T98G xenograft mice. T98G cells and human cervix carcinoma HeLa cell tumor from xenograft mice were used as standard and positive controls of *Alu*-repeat. Amplification of the human *Alu*-repeat sequence was not detected in the mouse spleen with leukemic cells, suggesting that mechanisms other than the insertion of human oncogenes were involved in the development of secondary leukemia (Figure 2c). Although the T98G-DDS10 tumor cells injected in the mice were present for a short period (Figure 1c), this affected the host cells and caused leukemia. We hypothesize that the cytokines secreted by T98G-DDS10 may be involved in the development of leukemia. Increased expressions of inflammatory cytokines, such as IL-4 and IL-6, often participate in the development of leukemia.[8–10] Thus, we examined the expression of these inflammatory cytokines in T98G-DDS10 cells compared with the original T98G cells by quantitative real-time PCR analysis. As expected, significant upregulation of h*IL-4* and h*IL-6* mRNA expression in T98G-DDS10 cells was observed compared with the original T98G cells (Figure 2d). These results suggest that IL-4 and IL-6, but not insertion of human DNA, partially contributed to the development of $CD3^+$ T-cell leukemia.

On the basis of these observations, we drew the conclusion that long-term hypoxia and nutrient starvation stress cycles to primary tumor cells can consequently stimulate leukemogenesis independent of leukemogenic genetic chromosome aberrations and mutation events by chemotherapy and radiotherapy. Secondary leukemia, such as acute lymphocytic leukemia, acute myeloid leukemia or myelodysplastic syndrome, is reported to occur in patients with a history of chemotherapy or radiotherapy of a primary solid tumor.[1,7] Our leukemic model is independent of chemotherapy, but dependent on hypoxia and nutrient starvation double stress. However, it is possible that tumor cells located far from the existing vasculature (central area of tumor mass) after chemo and radiotherapy undergo hypoxia and nutrient starvation because of disruption of the tumor vasculature. Expression of inflammation cytokines such as IL-6 has been reported to be upregulated following chemo and radiotherapy.[10] Thus, hypoxia and nutrient starvation in tumor microenvironments concomitant with the induction of inflammation cytokines in tumors may be important factors inducing the development of secondary leukemia. However, infusion of human cytokines into nude mice may not be sufficient for the development of T-cell leukemia, and other secretion factors or cell surface molecules may also be involved in this process.

Antiangiogenesis suppressing the supply of both oxygen and nutrients is an effective cancer treatment; however, after a long-term treatment, tumors may become aggressive[4–6] and may also affect host cells following antiangiogenesis, as shown in our mouse models. To date, there is no clinical evidence of secondary leukemia following antiangiogenesis therapy; however, many newly developed antiangiogenic inhibitors are currently undergoing clinical trials, which may show more effective antiangiogenic and antitumor effects. To further improve antiangiogenic therapy to overcome secondary problems, one approach is to eliminate double stress-resistant cancer

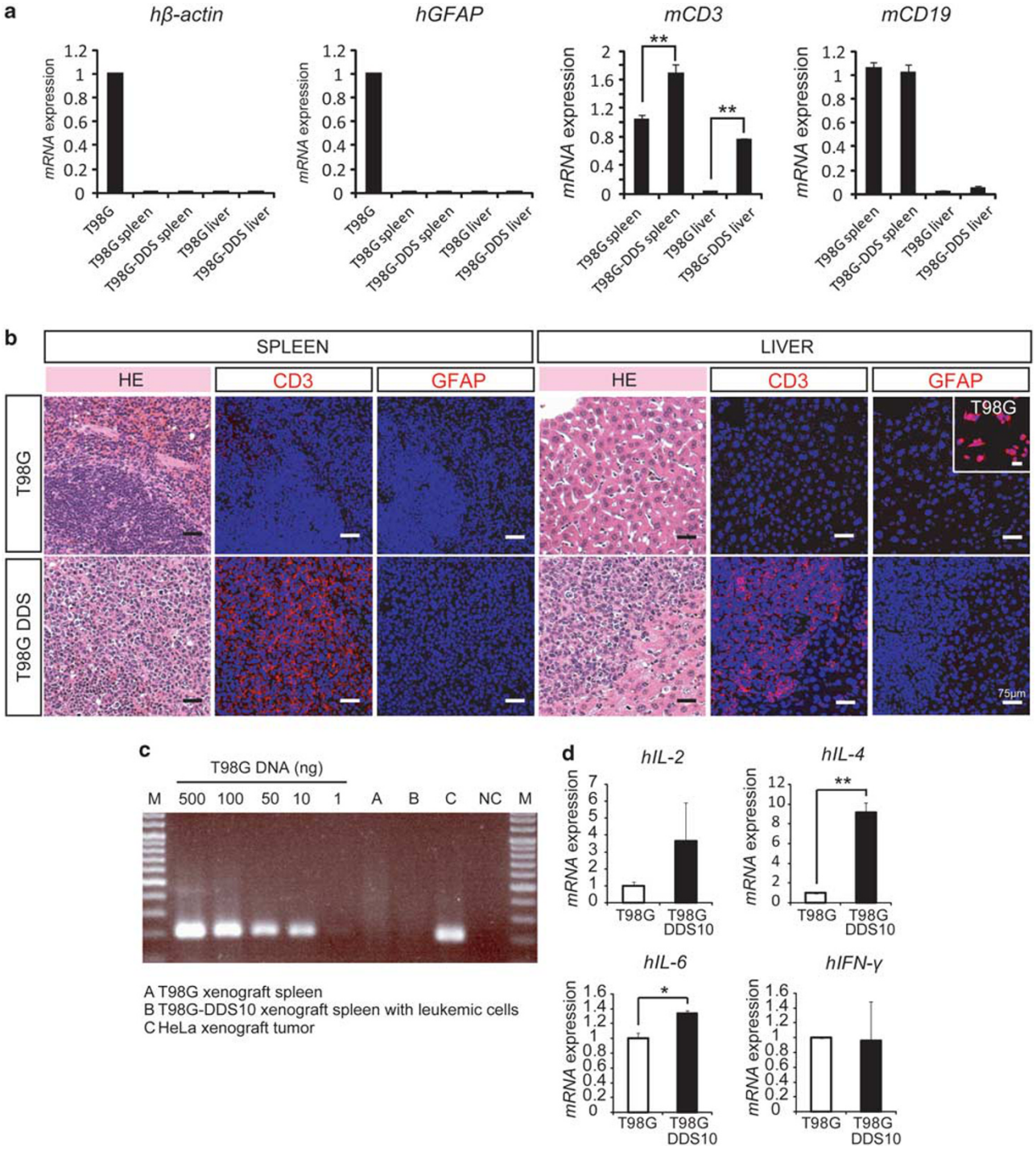

Figure 2 Upregulation of IL-4 and IL-6 gene expression in T98G-DDS10 cells and development of CD3+ T-cell leukemia. (**a**) Quantitative real-time PCR analysis of secondary tumor tissue against human *β-actin*, human *GFAP*, mouse *CD3* and mouse *CD19* mRNAs. ($n=3$) (**$P<0.01$). complementary DNA obtained from T98G was used as a positive control for human mRNA. **(b)** HE staining and immunohistochemical staining of spleen and liver from original T98G and T98G-DDS xenograft mice against CD3 and GFAP antibodies (Red) with To-Pro-3 nuclear staining (Blue). **(c)** Secondary leukemia does not contain human DNA at detectable levels. Detection of human DNA using *Alu* PCR assay on agarose gel following 30–40 cycles of PCR amplification. DNA obtained from cultured T98G cells was used as standard control (using 1–500 ng DNA and amplified for 30 cycles). DNA from HeLa xenograft tumor was used as a positive control for the amplification of human DNA in spleen samples of T98G xenograft and T98G-DDS10 xenograft mice (using 50 ng DNA and amplified for 40 cycles). M, 100 bp marker; NC, negative control. **(d)** T98G significantly upregulates expression of inflammation cytokines such as IL-4 and IL-6 following long-term hypoxia and nutrient deprivation double stress. Quantitative real-time PCR analysis was carried out to examine the mRNA levels of h*IL-2*, h*IL-4*, h*IL-6* and h*IFN-γ* in T98G-DDS10 cells compared with the original T98G cells. (*$P<0.05$; **$P<0.01$).

cells to inhibit the recurrence of the primary tumor, and also to inhibit inflammatory cytokines to prevent secondary tumors following remission of the primary tumor.

Only a very limited number of animal models of leukemogenesis are available. Our model—in which aggressive tumor cells induced leukemia in mice, independent of radiation and

chemotherapy—can be considered a novel model system for characterizing the molecular mechanisms underlying leukemogenesis.

In conclusion, human glioblastoma cells survived long-term hypoxia and nutrient starvation participated in the induction of CD3+ T-cell secondary leukemia in mice after a complete remission of the primary tumor xenograft, and independent of chemo and radiation treatments. Taken together, our results indicate that activated cancer cells exposed to hypoxia and nutrient starvation after anticancer therapy can affect the host microenvironment. Thus, targeting cancer cells resistant to hypoxia and nutrient starvation is important to develop novel cancer therapies and also to prevent secondary leukemogenesis. Our animal model system may be useful for elucidating the mechanism underlying hematopoietic malignancies.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on Blood Cancer Journal website (http://www.nature.com/bcj)