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Inhibition of angiogenesis and induction of endothelial and tumor cell apoptosis by green tea in animal models of human high-grade non-Hodgkin's lymphoma

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Recent reports suggest that green tea consumption may prevent or delay the growth of human cancer, possibly by impairing tumor invasion and/or by an anti-angiogenic effect. In NOD/SCID mice transplanted intraperitoneally with human non-Hodgkin's lymphoma (NHL) cell lines, Namalwa, RAP1-EIO and HS-Sultan, green tea prevented 50% of Namalwa tumors (P = 0.0017 by log-rank) and significantly inhibited RAP1-EIO and HS-Sultan tumor growth. Notably, treatment with the chemotherapy drug cyclophosphamide at the maximum tolerable dose was unable to prevent Namalwa tumor occurrence. In the three models evaluated, the frequency of apoptotic endothelial and tumor cells was significantly increased in mice given green tea compared to controls. These results support further trials in NHL to evaluate whether green tea, alone or in combination with chemotherapy, may delay or prevent disease progression.
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Introduction

Epidemiological and laboratory studies have indicated that green tea may have antitumor activity, and that its consumption may reduce the incidence of cancer and metastases.[1–3] *In vitro*, green tea and its main flavonol, epigallocatechin-3-gallate (EGCG), have shown effects on different neoplastic cell lines. EGCG reduces proliferation and induces apoptosis of cancer cells,[1] impairs tumor invasion and nourishment by inhibition of urokinase,[4] matrix metalloproteinases (MMP)-2 and MMP-9[5] and inhibits PDGF signaling.[6] Furthermore, a recent study has indicated that *in vivo* green tea is an effective inhibitor of angiogenesis.[7] This effect is of particular interest, because the growth of most types of cancers,[8] possibly including hematological malignancies,[9–11] is dependent on the generation of new blood vessels. Some preclinical studies have demonstrated the efficacy of green tea in animal models of tumors other than hematopoietic malignancies,[1–3] and along this line we evaluated the effect of green tea in models of human high-grade B cell non-Hodgkin's lymphoma (NHL). To this aim, we have screened the engraftment potential of different human NHL lines in NOD/SCID mice. Although mice bearing the Nude or the SCID mutation have been extensively used to evaluate human malignancies *in vivo*, these strains have residual immunity that may limit post-transplant neoplastic cell growth, while the NOD/SCID strain appears to be more convenient for human leukemia/lymphoma xenotransplantation.[12] In our study, mice were transplanted intraperitoneally (i.p.) instead of subcutaneously (s.c.) to generate a disease more similar to human B cell NHL. Namalwa, RAP1-EIO and HS-Sultan lines were able to generate i.p. tumors in the injection site, and these tumors were measurable with a caliper in a rigorous way to evaluate the efficacy of different therapies. All of the cell lines evaluated in the present work had a similar phenotype ($CD3^-$, $CD10^+$, $CD13^-$, $CD19^+$, $GlyA^-$). Namalwa cells derive from an EBV^+ Burkitt's NHL. Although HS-Sultan has been described by some authors as a plasmacytoma or plasma cell dyscrasia line for its morphology, DNA fingerprinting has demonstrated its generation from an $EBNA^+$ Burkitt's NHL.[13] The newly established RAP1-EIO line has a t(14:18) translocation and is similarly representative of an aggressive human B cell NHL. Here, we report the effect of green tea administration on tumor growth in these preclinical models.

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Materials and methods

Cell lines

Namalwa and HS-Sultan lines were obtained from ATCC (Manassas, VA, USA). After informed consent was obtained, the RAP1-EIO cell line was established in 1998 from the bone marrow (BM) of a male 61-year-old patient who had a diagnosis of T cell-rich B cell NHL. Cells display a $CD3^-$, $CD10^+$, $CD13^-$, $CD19^+$, $GlyA^-$, sm/cy kappa$^+$, sm/cy lambda$^-$ phenotype and a t(14:18) karyotype after passage in the mouse.

Animal studies

Six- to 8-week-old NOD/SCID mice were injected i.p. with 10×10^6 Namalwa (n = 20), RAP1-EIO (n = 12) or HS-Sultan (n = 12) cells and evaluated every other day for tumor growth. Tumors were measured by calipers, and the method of Bohem *et al*[14] was used to calculate tumor volume. Green tea was prepared as described in detail by Wang *et al*.[15] Briefly, green tea leaves (12.5 g) were added to 500 ml purified boiling water and steeped for 15 min. The infusion was cooled to room temperature and filtered, then the procedure was repeated a second time. The resulting solution (1.25% green tea extract) is similar to tea brews consumed by humans and contains 708 μg/mL EGCG.[15] Green tea extracts were prepared every 3 days for animal use. Storage for 72 h in water bottles was found to be associated with $<$10% EGCG decrease.[1,15] Transplanted mice were randomly assigned to receive water or green tea as the sole drinking fluid. As recently reported,[7] following this approach the amount of green tea in mice drinking water generate EGCG plasma concentrations in the range of 0.1–0.3 μM which are observed in humans after drinking two to three cups of tea daily. In a separate study, six mice transplanted with Namalwa cells were given water as a drinking fluid and cyclophosphamide (CTX) i.p. at the maximum tolerable dose (MTD) of 75 mg/kg on days 3, 5 and 7 after transplantation. CTX doses higher than MTD were found to kill 60% of treated mice in the absence of a significantly higher response rate.

All procedures involving animals were done in accordance with national and international laws and policies. Tumor-bearing mice were sacrified by CO_2 inhalation. Tumor engraftment was confirmed by: (1) conventional histology (hematoxylin–eosin and Giemsa staining); (2) immunohistochemistry (IHC, 10% formalin-fixed and paraffin-embedded samples immunostained with the anti-CD10 and CD20 monoclonal antibodies from DAKO (Glostrup, Denmark); and (3) flow cytometry (FC, evaluation of human CD19 and CD20 antigens from Becton Dickinson (Mountain View, CA, USA) monoclonal antibodies).

Angiogenesis in solid tumors was evaluated by FC using monoclonal antibodies against murine CD31 (clone MEC 13.3; PharMingen, San Diego, CA, USA) and murine CD34 (clone RAM34; PharMingen). In FC studies, tumors were dissolved at the single-cell level, and $100–500 \times 10^3$ cells were incubated at 22 °C for 30 min in PBS-1% BSA with monoclonal antibodies. By means of FACScalibur (Becton Dickinson), the percent of stained cells was determined as compared to PE- or FITC-conjugated isotypic control. A portion of each sample was incubated with the appropriate isotype control antibodies to establish the background level of non-specific staining, and positivity was defined as being greater than non-specific background staining. Endothelial cells were enumerated as murine $CD31^+CD34^+$ cells. According to the method of Philpott *et al*,[16] 7AAD and annexin V were used in FC evaluations to depict apoptotic and dead cells. Using this approach, early apoptotic cells were depicted by the binding of annexin V, and progression of apoptosis was detected by 7AAD accumulation. Microvessel density (MVD) was evaluated by light microscopy as previously described.[10] In brief, hematoxylin and eosin stained slides were evaluated at 40 × and 100 × to detect the area with the highest MVD (hot spot). Three microscopic fields were then examined in this area at 250 × magnification (each field representing an area of 0.72 mm^2), and the mean MVD value was recorded. Any endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered a single, countable microvessel.

Green tea-treated mice without visible signs of tumor growth were sacrified when tumor growth was observed in all control animals. In these negative mice, autopsy was performed to further investigate by means of light microscopy, FC and IHC the presence of solid tumors and/or human cells in the PB and BM.

Toxicity of chronic green tea administration to NOD/SCID mice was evaluated in six non-transplanted animals given green tea as the sole drinking fluid for 120 days.

Statistical analysis

Statistical comparisons were performed using the *t*-test and analysis of variance (ANOVA) when data were normally distributed and the non-parametric analyses of Spearman and Mann–Whitney when data were not normally distributed. The log-rank test was used to compare event-free survival (EFS) between different groups. Values of P lower than 0.05 were considered as statistically significant.

Results

All of the control (ie water-recipient) mice transplanted with Namalwa, RAP1-EIO or HS-Sultan cells developed i.p. measurable tumors in the injection site within 30 days (Figures 1–3), thus indicating that the NHL models described in this study had 100% engraftment efficiency in the absence of therapy. In mice transplanted with Namalwa, RAP1-EIO or HS-Sultan cell lines, tumors grew as solid masses in the peritoneal cavity infiltrating the peritoneum of the abdominal wall. Tumors were composed of large cells growing in a diffuse pattern, apoptotic bodies and large areas of necrosis were detectable. Tumor metastasis in sites different from that of injection was not observed during the first month after transplantation. When some mice were transplanted and left untreated to evaluate tumor spreading, metastasis to regional lymph nodes and multiple visceral organs was observed during the second month after transplantation. In a few cases, ascites containing tumor cells were observed in HS-Sultan transplanted mice left untreated for more than 30 days.

Remarkably, 50% of mice transplanted with Namalwa cells and treated with green tea did not develop visible tumors (n = 10 per arm, P = 0.0017 by log-rank *vs* control, Figure 2a). To confirm the absence of tumor development, these negative mice were sacrified on day 35 and found to be free of tumor by autopsy. In mice transplanted with Namalwa cells, green tea inhibited both tumor take (as shown by event-free survival rates in Figure 2a) and tumor growth rate. In fact, in the mice who developed a Namalwa tumor despite green tea treatment, tumor inhibition on day 30 was 43% (Figure 2b). In order to compare the therapeutic potential of green tea with that of a chemotherapy drug usually administered to NHL patients, six Namalwa-transplanted mice were given CTX at the MTD. Although in CTX-treated mice the extent of tumor growth was delayed when compared to controls, all of the mice developed a visible and measurable tumor within 30 days (Figure 2c). In mice transplanted with RAP1-EIO and HS-Sultan cells, tumor growth was significantly inhibited by green tea administration (n = 6 per arm). In RAP1-EIO-transplanted mice, tumor inhibition ranged from 82% on day 10 to 39%

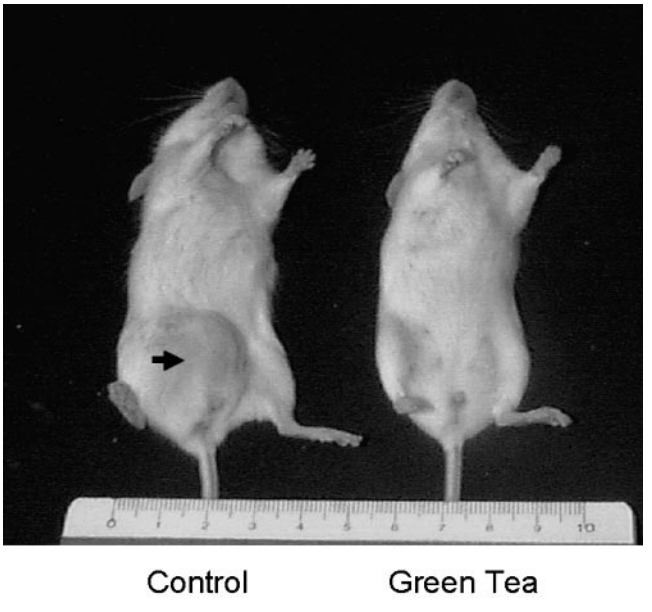

Figure 1 Extent of tumor growth in NOD/SCID mice transplanted i.p. with 10×10^6 Namalwa cells. A visible and measurable tumor growing in the injection site is indicated by the arrow in the control mouse on the left. Conversely, tumor is absent in the mouse on the right which received green tea as the sole drinking fluid.

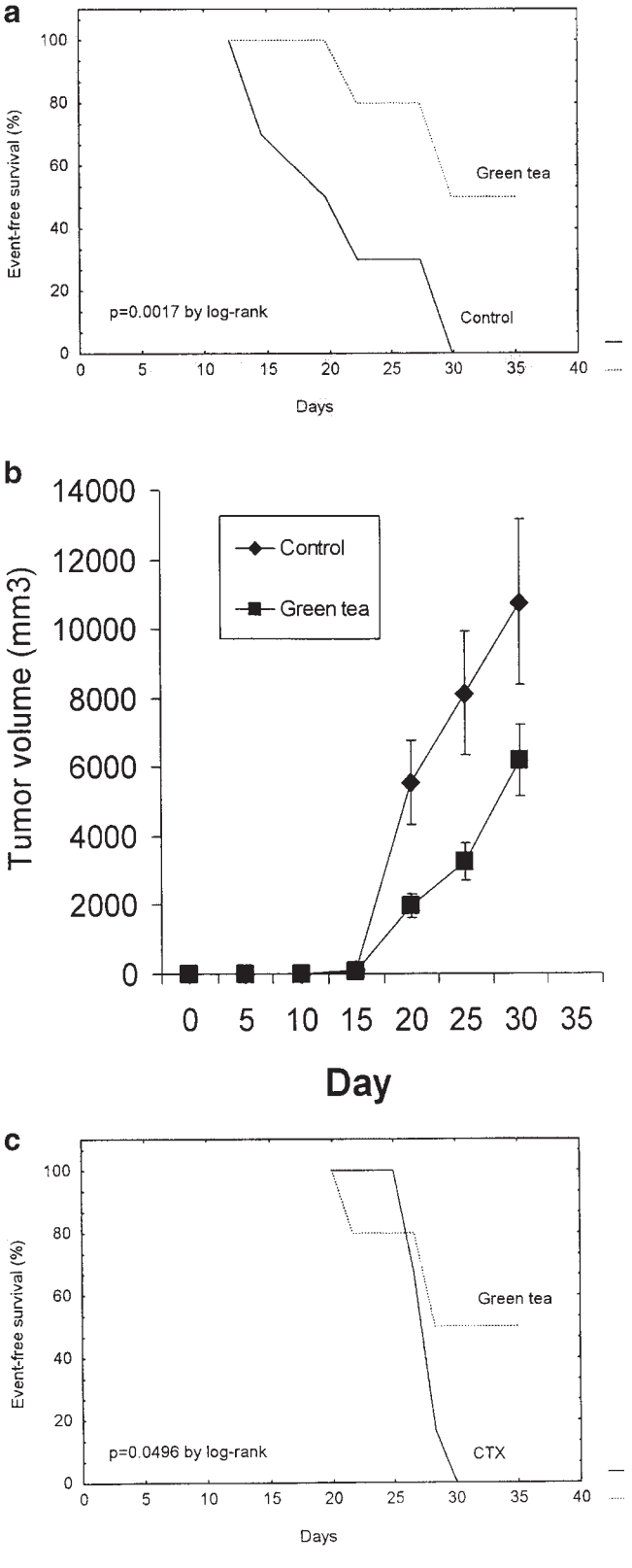

Figure 2 (a) Event-free survival in NOD/SCID mice transplanted with Namalwa cells and given water or green tea as drinking fluid (n = 10 per arm). (b) Tumor growth in the mice who developed a Namalwa tumor despite green tea treatment and in controls, green tea-mediated tumor inhibition on day 30 was 43%. (c) Event-free survival in NOD/SCID mice transplanted with Namalwa cells. Data from six mice given water and treated with CTX i.p. at the maximum tolerable dose (MTD) of 75 mg/kg on days 3, 5 and 7 after transplantation are compared with data from 10 mice given green tea.

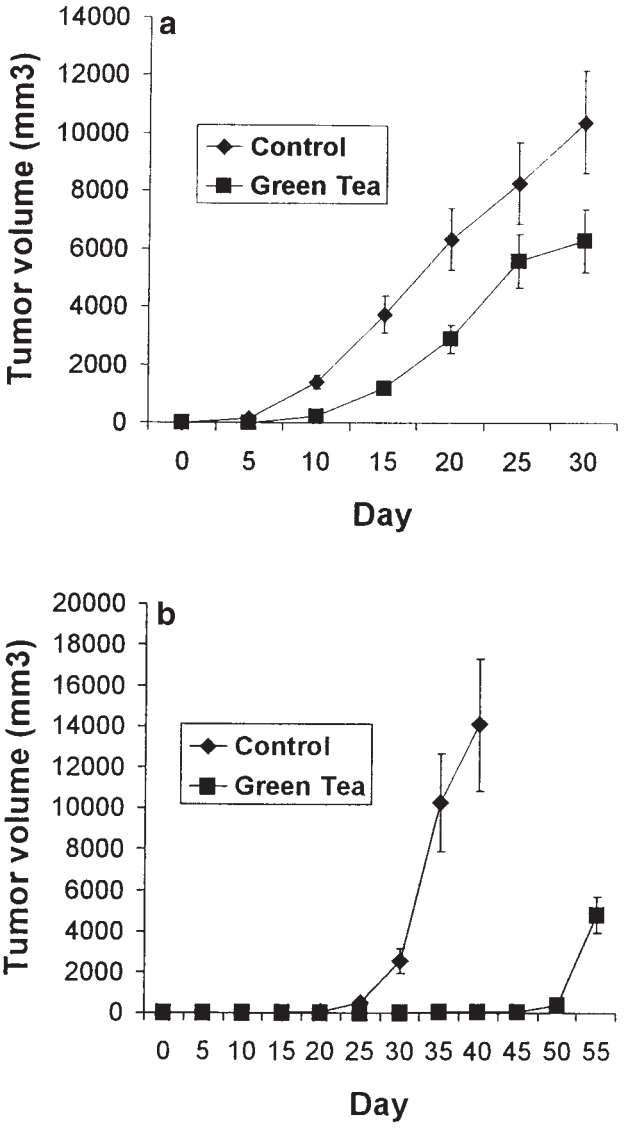

Figure 3 Green tea treatment of NOD/SCID mice transplanted i.p. with (a) 10×10^6 RAP1-EIO cells and (b) 10×10^6 HS-Sultan cells. n = 6 per arm; results are reported as mean ± 1 s.d. of tumor volume.

on day 30 (Figure 3a). In HS-Sultan transplanted mice, mean tumor inhibition was higher than 95% (Figure 3b).

We have recently reported that the frequency of murine endothelial $CD31^+$ $CD31^+$ cells correlates with MVD in NOD/SCID mice transplanted with human primary leukemia/lymphoma cells and cell lines.[17] In the present studies, MVD was only slightly reduced in mice given green tea compared to controls (Table 1). However, as shown by 7AAD and annexin V expression, in green tea-treated animals the frequency of both tumor (Figure 4) and endothelial (Figure 5) apoptotic cells was significantly increased.

Six mice were not transplanted with NHL cells and given green tea as the sole drinking fluid for 120 days to assess toxicity. At autopsy, liver, kidney, bladder, stomach, gut, lung, hearth, brain, spleen or BM toxicity was not observed.

Discussion

In the past, most mouse models of human NHL involved s.c. infusion of neoplastic cells. Taking advantage of the enhanced

Table 1 Tumor MVD and frequency of tumor (human CD19$^+$) and endothelial (mouse CD31$^+$CD34$^+$) apoptotic cells detected as 7AAD positive by FC evaluation

Transplanted cell line		*n*	*Human CD19$^+$ 7AAD$^+$ cells (%)*	*Mouse CD31$^+$ CD34$^+$ 7AAD$^+$ cells (%)*	*MVD*
Namalwa	Control	10	23 ± 8	8 ± 3	17 ± 2
	Treated	10	54 ± 18*	12 ± 4*	16 ± 1
RAP1-EIO	Control	6	10 ± 8	3 ± 2	26 ± 8
	Treated	6	35 ± 19*	12 ± 4*	22 ± 5
HS-Sultan	Control	6	18 ± 3	4 ± 2	34 ± 3
	Treated	6	41 ± 11*	10 ± 3*	33 ± 2

*$P < 0.05$ *vs* control.

immunosuppression of NOD/SCID mice compared to other strains,[12] we have generated reliable *in vivo* models of aggressive i.p. human B cell NHLs which are measurable, more similar to the human disease and have 100% engraftment efficiency. In this study, we have found that green tea has a remarkable effect on the growth of three different cell lines, including prevention of 50% of tumors in mice transplanted with Namalwa cells. This line is of particular interest because it has been recently found to be the most aggressive one in a panel of lymphoid lines tested in s.c. xenotransplant studies.[12] Moreover, in the present work, all of the Namalwa-transplanted mice developed tumors in spite of CTX treatment at the MTD.

Recent papers by us,[17,18] and others,[19–21] have indicated a possible role of angiogenesis and angiogenic growth factors in NHL. Expression of vascular endothelial growth factor (VEGF) and its receptors has been reported in B cell hematopoietic malignancies,[17,20] and the autocrine and paracrine roles of this growth factor in NHL are under evaluation. Furthermore, high circulating levels of VEGF[18,19] and of the other endothelial cell mitogenic factor basic-fibroblast growth factor (b-FGF, Refs 18, 21) have been found to correlate with poor prognosis in NHL. In animal models of human cancer, one should consider that tumor angiogenic phenotype can be modulated by cytokines released by host cells in specific microenvironments. Human renal cancer cells, for instance, express high b-FGF levels when growing in kidney, but not when growing s.c.[22] Data from our i.p. human NHL models support previous findings about prevention of cancer by green tea consumption[1] and anti-angiogenic properties of green tea.[7] It should be noted that, until recently, it was thought that anti-angiogenic drugs operate only after tumor neovascularization has occurred. Li *et al*[23] have demonstrated that an anti-angiogenic drug can function before a tumor has generated new blood vessels. As commented by Folkman,[24] this novel finding provides a suitable conceptual framework for the anti-angiogenic action of EGCG and green tea in the prevention of human tumors.

Furthermore, similarly to what has been described in mouse models of anti-angiogenic therapies of epithelial tumors,[25,26] we have observed an enhanced frequency of apoptotic endo-

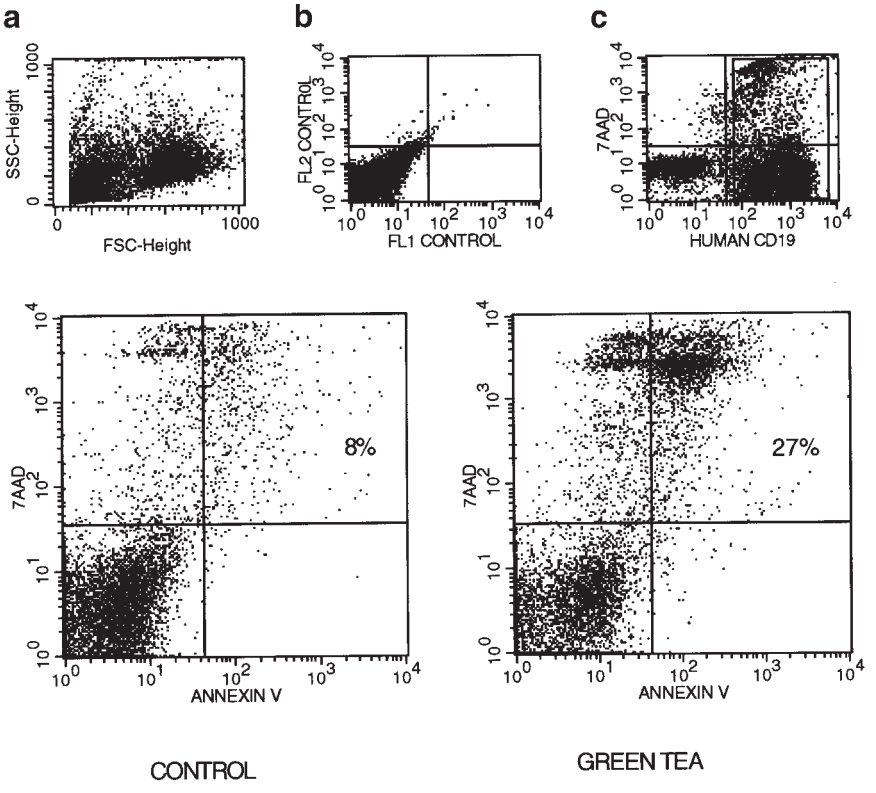

Figure 4 Representative dot plots indicating the frequency of tumor cell apoptosis in RAP1-EIO-transplanted animals. Solid tumors were dissolved at the single cell level and evaluated by flow cytometry. Panel a shows forward and side scatters of the cell suspension, panel b the negative control and panel c the analysis gate on human CD19$^+$ cells. As indicated by the percentage of 7AAD$^+$/annexin V$^+$ cells in the bottom panels, the frequency of CD19$^+$ apoptotic cells was significantly increased in mice given green tea.

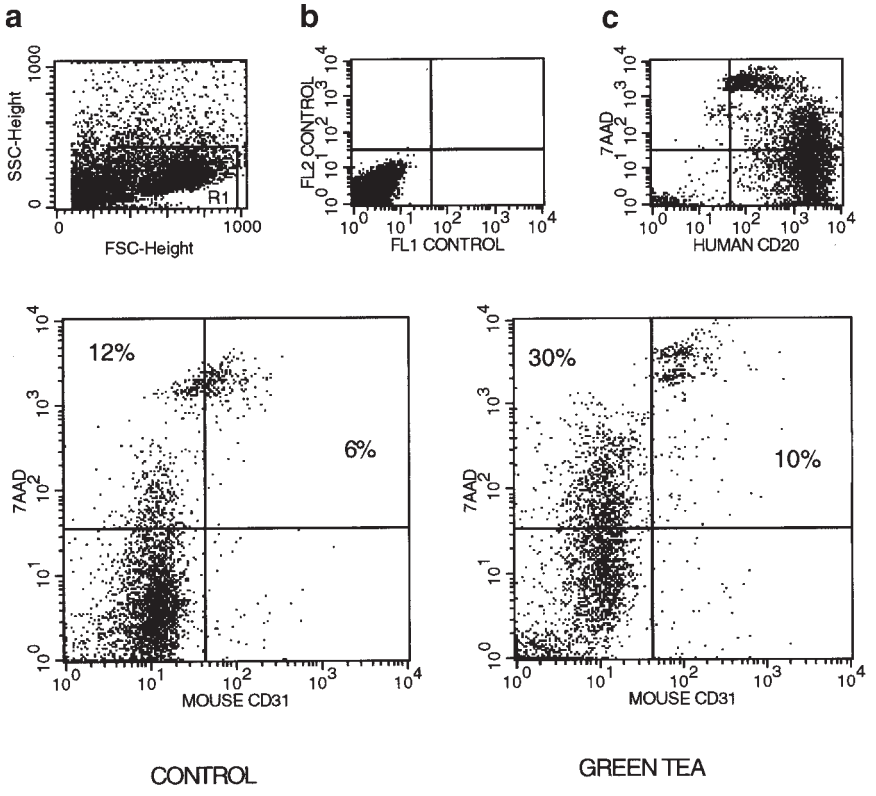

Figure 5 Representative dot plots indicating the frequency of endothelial cell apoptosis in HS-Sultan-transplanted animals. Tumors dissolved as single cells were evaluated by flow cytometry. Panel a shows forward and side scatters of the cell suspension and the analysis gate, panel b the negative control and panel c the human $CD20^+$ phenotype of the tumor. As indicated by the percentage of murine $CD31^+/7AAD^+$ cells in the bottom panels, the frequency of endothelial apoptotic cells was significantly increased in mice given green tea.

thelial cells in the tumors of green tea-treated animals. The evaluation of apoptotic tumor and endothelial cells in human xenograft by three-color FC is a novel quantitative measurement. We have recently found in NOD/SCID mice transplanted with a panel of 12 human leukemia/lymphoma cells that the frequency of murine endothelial $CD31^+CD34^+$ cells in xenograft tumors correlates with tumor engraftment potential, speed of engraftment (inverse correlation) and the frequency of apoptotic tumor cells (inverse correlation, Ref. 17). Interestingly, in this model these parameters correlated better with the frequency of $CD31^+$ $CD34^+$ cells than with traditional MVD evaluation by microscopy.[17] In the present study, we have also observed an increased frequency of tumor apoptotic cells in green tea-treated animals, and it remains to be fully clarified whether this is due to the anti-angiogenic effect of green tea or to its direct effect on tumor cells. In fact, an EGCG inhibitory effect on epithelial tumor cell lines without action on normal epithelial cells has been reported in the past.[27] We are currently evaluating whether green tea inhibits the growth of endothelial cells to a greater extent than tumor cells.

In conclusion, our data support the efficacy of green tea in aggressive B cell NHL. It is of interest that the anti-angiogenic drug thalidomide has been found to be effective in chemotherapy-refractory patients with multiple myeloma (MM), another B cell-related malignancy.[28] Furthermore, very recent abstracts have indicated that thalidomide seems to be also effective in myelodysplastic syndromes, myeloproliferative disorders and myelofibrosis.[29,30] These hematological diseases, like MM, are associated with relevant BM angiogenesis.[10,11] It should be considered that anti-angiogenic therapy may not be able to induce tumor regression, but could induce tumor dormancy or stabilization.[31] In this context, green tea fits well with the required characteristics of anti-angiogenic drugs, because it has a direct effect on newly generated endothelial cells and can be administered for long periods without toxicity. Since green tea-related compounds have already entered phase I studies,[32,33] it would be useful to design clinical trials in NHL patients to evaluate whether green tea, alone or in combination with chemotherapy, could delay or prevent disease progression.

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