Anticancer Therapies Combining Antiangiogenic and Tumor Cell Cytotoxic Effects Reduce the Tumor Stem-Like Cell Fraction in Glioma Xenograft Tumors

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Abstract

Vascular endothelial cells have been identified as a critical component of the neural stem cell niche, raising the possibility that brain tumor stem-like cells (TSLC) may also rely on signaling interactions with nearby tumor vasculature to maintain their stem-like state. The disruption of such a TSLC vascular niche by an antiangiogenic therapy could result in loss of stemness characteristics associated with intrinsic drug resistance and, thus, preferentially sensitize TSLC to the effects of chemotherapy. Considering these possibilities, we investigated the impact of antiangiogenic anticancer therapy on the TSLC fraction of glioma tumors. Athymic nude mice bearing s.c. tumor xenografts of the C6 rat glioma cell line were treated with either a targeted antiangiogenic agent, antiangiogenic schedules of low-dose metronomic chemotherapy, combination therapies of antiangiogenic agents and chemotherapy, or, for the purpose of comparison, a conventional cytotoxic schedule of maximum tolerated dose chemotherapy using cyclophosphamide. Targeted antiangiogenic therapy or cytotoxic chemotherapy did not reduce the fraction of tumor sphere-forming units (SFU) in the tumor, whereas all treatment groups that combined both antiangiogenic and cytotoxic drug effects caused a significant reduction in SFU. This work highlights the possibility that selective eradication of TSLC may be achieved by targeting the tumor microenvironment (and potentially a supportive TSLC niche) rather than the TSLC directly. Furthermore, this work suggests a possible novel effect of antiangiogenic therapy, namely, as a chemosensitizer of TSLC, and thus represents a possible new mechanism to explain the ability of antiangiogenic therapy to enhance the efficacy of chemotherapy. [Cancer Res 2007;67(8):3560–4]

Introduction

Numerous studies indicate that the growth of gliomas (1–4), and a number of other types of cancer (5), is initiated and driven by a subpopulation of cancer cells with stem-like characteristics, including self-renewal capacity and the ability to differentiate. It has been postulated that, in addition to these hallmarks of "stemness," tumor stem-like cells (TSLC) may also possess a number of other properties associated with normal stem cells, including a slow proliferation rate, active DNA damage repair and antiapoptotic pathways, and the expression of multidrug transporters on the plasma membrane (6). Indeed, it has been shown that glioma cells exhibiting the side-population phenotype, which is characterized by cellular exclusion of the dye Hoechst 33342 primarily attributed to the ABCG2 multidrug transporter, are enriched with TSLC (4). Furthermore, $CD133^+$ glioma stem-like cells have been found to resist doses of radiation lethal to surrounding non–stem-like cells in the tumor by preferential activation of the DNA damage response (7). The retention of such properties by TSLC suggests that, like normal stem cells, TSLC may be inherently resistant to many traditional anticancer therapies that target rapidly dividing cells. This represents a daunting therapeutic challenge because the characterization of the TSLC as the proliferative driving force in the tumor suggests that TSLC must be eradicated to permanently or significantly arrest tumor growth. As such, there is growing interest in developing therapeutic strategies specifically aimed at eliminating or affecting the TSLC population.

Given the demonstrated similarities between neural stem cells and glioma TSLC, it stands to reason that glioma TSLC may also, like neural stem cells, exist within a supportive niche. Through contact-mediated and paracrine signaling interactions between stem cells and the niche microenvironment, the niche maintains and controls critical stem cell properties and functions (8). Soluble factors secreted by vascular endothelial cells have been found to promote self-renewal and inhibit differentiation of neural stem cells, suggesting that neural stem cells have a vascular niche (9). This finding raises the possibility that glioma TSLC may also rely on interaction with a vascular niche to maintain their stem-like properties, and consequently, their ability to drive tumor growth. Indeed, a very recent study provides compelling evidence that brain TSLC are supported by a vascular niche (10). Consistent with this notion, many studies have found that proangiogenic growth factors such as basic fibroblast growth factor (bFGF; refs. 1–4), epidermal growth factor (EGF; refs. 1–3) and platelet-derived growth factor (4), which would ostensibly be in higher concentration near blood vessels, permit maintenance and expansion of glioma TSLC in culture. Considering the possibility that glioma TSLC are maintained by a supportive vascular niche, we investigated whether antiangiogenic therapies can reduce the TSLC fraction of glioma tumors.

Materials and Methods

Cell culture. C6 (American Type Culture Collection, Manassas, VA) were maintained in DMEM with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT). Human umbilical vein endothelial cells (HUVEC; Cambrex Bio Science Walkersville, Inc., Walkersville, MD) were maintained in 1% gelatin-coated dishes (Nalgene Nunc Int., Rochester, NY) in MCDB131 (JRH Biosciences, Lenexa, KS) with L-glutamine (2 mmol/L; Invitrogen, Burlington, Ontario, Canada), heparin (10 units/mL; Pharmaceutical

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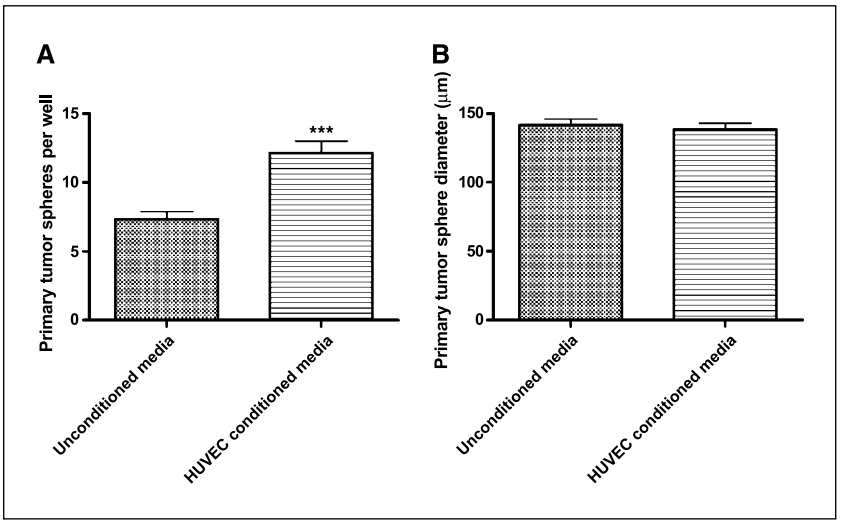

Figure 1. Primary tumor sphere-forming capacity of C6 glioma following culture in HUVEC-conditioned media. C6 glioma cells were grown as monolayers in either DMEM + 10% FBS (*Unconditioned media*), or DMEM + 10% FBS that had been incubated with subconfluent HUVEC for 24 h (*HUVEC conditioned media*). Following 7 d of culture in either condition, C6 cells were trypsinized, washed with PBS, and subjected to primary tumor sphere-forming assay as described in Materials and Methods. After 11 d, the number of spheres per well was enumerated (*A*), and the diameter of at least 50 spheres per group was measured (*B*). Results represented as mean ± SEM. ***, $P < 0.0001$ (Student's *t* test).

Partners of Canada, Inc., Richmond Hill, Ontario, Canada), vascular endothelial growth factor (VEGF; 10 ng/mL; R&D Systems, Minneapolis, MN), EGF (10 ng/mL; R&D Systems), and bFGF (5 ng/mL; R&D Systems). All cells were grown in a humidified incubator at 37°C in 5% CO_2 and harvested with 0.25% trypsin-0.03% EDTA (Invitrogen).

Generation of HUVEC conditioned media. Eighty percent confluent 14-cm dishes of HUVEC were washed with PBS and incubated in 12 mL DMEM with 10% FBS for 24 h. Conditioned media was centrifuged 7 min at 1,000 rpm, and supernatant was filter sterilized (0.2-μm pore PES membrane, Nalgene Nunc Int.).

Mice, rat tumor xenografts, and treatment regimens. Adult female athymic nude mice (Harlan, Indianapolis, IN) were injected s.c. with 10^6 C6 cells in 200 μL serum-free DMEM, and the following treatments were initiated 11 days later: low-dose metronomic (LDM) cyclophosphamide, 20 mg/kg/day p.o. via the drinking water, as described (11); maximum tolerated dose (MTD) cyclophosphamide, 100 mg/kg i.p. on days 1, 3, and 5 of a 21-day cycle; DC101, a rat anti-mouse VEGF receptor 2 (VEGFR)/flk-1 monoclonal antibody (12), 800 μg mouse i.p. every 3 days; combined LDM cyclophosphamide + DC101; combined MTD cyclophosphamide + DC101; or bolus + LDM cyclophosphamide, 100 mg/kg i.p. on day 1 and 20 mg/kg/day p.o. on days 3 to 21, of a 21-day cycle. Each group received normal saline p.o. and i.p. corresponding to p.o. and i.p. cyclophosphamide doses, and PBS i.p. corresponding to DC101 doses, corresponding to all treatment schedules which that group was not receiving. Tumors were measured using Vernier calipers, calculating volume as: width^2 × length × 0.5. After 2 weeks of treatment, tumors were excised and weighed.

Sphere-forming assays. Cultures were trypsinized and strained through a 40-μm sieve. Tumors were minced, digested in 4 mg/mL collagenase 3 (Worthington, Freehold, NJ), 2 mg/mL hyaluronidase (Sigma-Aldrich, Oakville, Ontario, Canada), and 2 mg/mL collagenase IV (Sigma) in serum-free DMEM for 25 min at 37°C, strained through a 40-μm sieve, incubated 5 min at room temperature in 0.75% NH_4Cl to lyse RBC, and washed with DMEM + 10% FBS. Single cell suspensions were confirmed microscopically, washed with PBS, and suspended at 1,000 cells/mL in serum-free DMEM supplemented as described (4). One hundred microliters of the cell suspension was plated in each of the 30 wells of a 96-well microplate (Nunc). Each well was fed 25 μL serum-free supplemented DMEM every other day for 11 days (from culture) or 14 days (from tumor). Spheres (tight, spherical, nonadherent masses >90 μm in diameter) per well were counted, and at least 50 spheres per group were measured with an ocular micrometer. For secondary sphere-forming assays, primary spheres were dissociated mechanically and processed as in the primary assay.

Circulating endothelial progenitor cell analysis. Blood was collected by retro-orbital bleed following treatment, and CEP levels were evaluated using four-color flow cytometry as described (13).

Results

Factors secreted by endothelial cells enhance the capacity of glioma cells to form tumor spheres *in vitro*. The C6 rat glioma cell line, which is known to contain a subpopulation of TSLC (4), was grown in either normal serum-containing media or HUVEC-conditioned serum-containing media. After 7 days, the cells in each culture were subjected to an *in vitro* primary tumor sphere-forming assay at clonal density (1,000 cells/mL). It has been shown in C6 (4) and primary human glioma cultures (2, 3) that TSLC, but not non–stem-like tumor cells, form primary tumor spheres in permissive culture conditions. Furthermore, we have found that serum-free C6 cultures enriched in sphere-forming units (SFU; compared with serum-containing C6 cultures) are also enriched in tumor initiating cells, as determined by limiting dilution transplant into immune-deficient mice.[4] We have also shown that the number of SFU within primary C6 tumor spheres expands in a secondary sphere-forming assay (see Fig. 4*A* and *C*), and prolonged passaging of C6 tumor spheres has been found to selectively enrich the TSLC fraction (4). Taken together, these observations indicate that C6 primary tumor spheres are self-renewing, and that their incidence is correlated with the presence of TSLC. Therefore, we used performance in the primary sphere-forming assay as a surrogate readout of the TSLC fraction in our *in vitro* cultures and *in vivo* tumors. C6 cultures grown in HUVEC conditioned media formed significantly more spheres (of similar size) compared with C6 grown in unconditioned identical media (Fig. 1). This suggests that endothelial cells secrete soluble factors that support and/or enhance the high proliferative potential (and presumably, by extension, tumorigenic properties) of glioma TSLC. Taken together with evidence in the literature that human brain TSLC are maintained by a vascular niche (10), and that C6 TSLC require angiogenic growth factors for maintenance and expansion in culture (4), our finding supports the notion that a vascular niche contributes to the maintenance and control of TSLC function in the C6 glioma model.

Therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the number of tumor sphere-forming cells in glioma xenografts. To investigate the effects of antiangiogenic

[4] C. Folkins, unpublished observations.

therapies on the glioma TSLC fraction, we treated nude mice bearing s.c. C6 tumor xenografts with several therapeutic strategies. These included the mouse VEGFR2 targeting antibody DC101 (12), daily LDM cyclophosphamide, which is thought to work in large part by an antiangiogenic mechanism (14), and an alternative schedule of LDM cyclophosphamide that includes an upfront bolus dose of cyclophosphamide (bolus + LDM cyclophosphamide) and also works by an antiangiogenic mechanism (15). For the purpose of comparison, we also included a group treated with a conventional cytotoxic MTD schedule of cyclophosphamide. In addition, we included groups treated with a combination of DC101 with either LDM cyclophosphamide or MTD cyclophosphamide because combinatorial therapies of antiangiogenic agents with

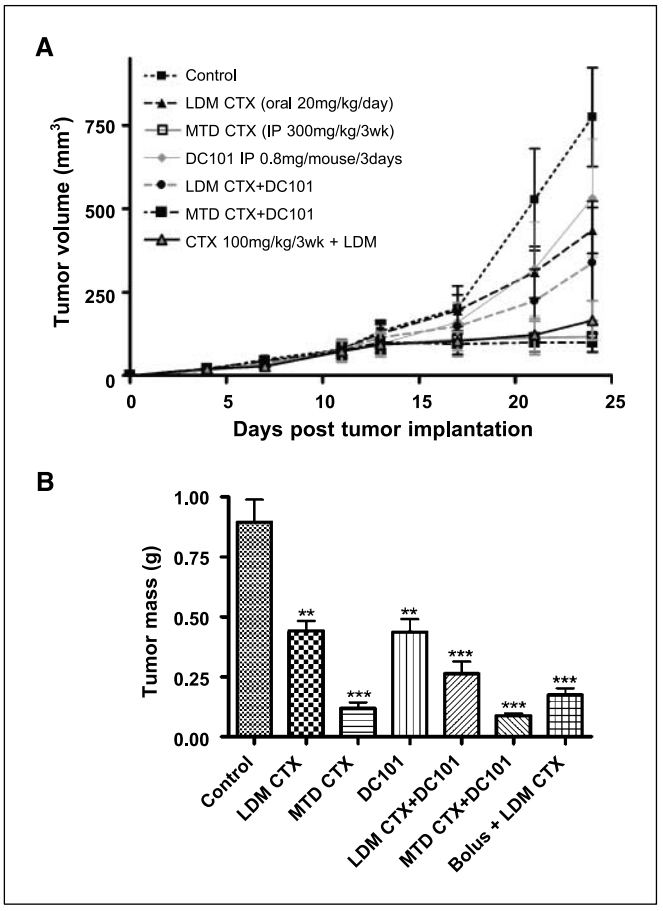

Figure 2. Treatment of mice bearing s.c. C6 tumor xenografts with various antiangiogenic, cytotoxic, and combination antiangiogenic plus cytotoxic therapies. A total of 49 female athymic nude mice were implanted with 10^6 C6 glioma cells s.c. in the right flank. Eleven days following implantation, mice were divided into seven treatment groups containing seven mice each, and treatments were initiated. Mice were treated with either LDM cyclophosphamide (*CTX*), 20 mg/kg/day given continuously through the drinking water, MTD cyclophosphamide, 100 mg/kg i.p. on days 1, 3, and 5 of a 21-d cycle, DC101, 800 μg/mouse i.p. every 3 d, combined LDM cyclophosphamide + DC101, combined MTD cyclophosphamide + DC101, or bolus + LDM cyclophosphamide, 100 mg/kg i.p. on day 1, and 20 mg/kg/day continuous p.o. administration on days 3 to 21 of a 21-d cycle. Treatment was continued for 2 wks, and tumor size was measured periodically using Vernier calipers (*A*). Tumors were excised and weighed at the end of the treatment period (*B*). One mouse from the LDM cyclophosphamide treatment group was found dead during the treatment period, and one from the MTD cyclophosphamide + DC101 group was sacrificed due to signs of neurologic impairment. Loss of these mice did not seem to be related to either the tumor xenograft or the therapy, and they were excluded from analysis. Results represented as mean ± SD (*A*) and mean ± SEM (*B*). **, $P = 0.0015$ (LDM cyclophosphamide); **, $P = 0.0011$ (DC101); ***, $P < 0.0001$ versus control (Student's *t* test).

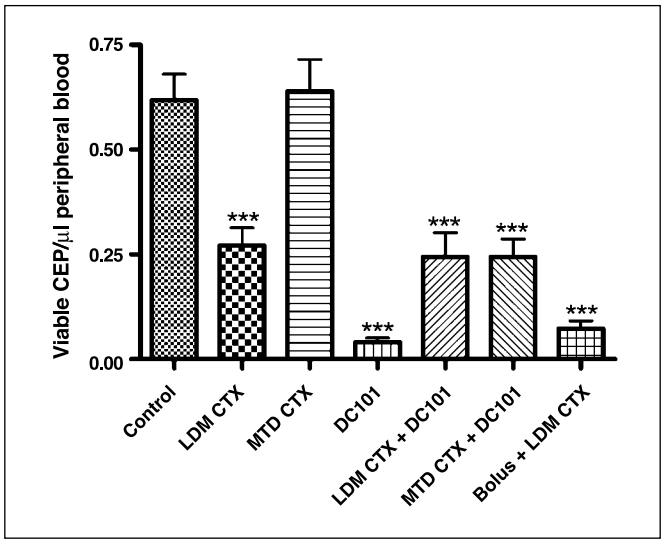

Figure 3. Peripheral blood levels of CEP cells in mice bearing s.c. C6 tumor xenografts following treatment. Following 2 wks of treatment with a variety of therapies (see Fig. 2), ~200 μL of blood was collected from each mouse by retro-orbital bleed. Levels of CEP cells were enumerated by four-color flow cytometry, as described in Materials and Methods. Results represented as mean ± SEM. ***, $P = 0.001$ (LDM cyclophosphamide, LDM cyclophosphamide + DC101); ***, $P < 0.0001$ (DC101, bolus + LDM cyclophosphamide); ***, $P = 0.0006$ (MTD cyclophosphamide + DC101) versus control (Student's *t* test).

chemotherapy are frequently superior to either monotherapy. Indeed, as monotherapies, antiangiogenic drugs such as bevacizumab seem to have very limited therapeutic efficacy in the treatment of advanced-stage cancers (16). After a delay period to allow for tumor establishment, animals were treated for 14 days. Tumor volumes were assessed periodically by caliper measurement (Fig. 2*A*). Following the treatment period, tumors were harvested and weighed (Fig. 2*B*). At the end of the treatment period, there were no significant differences in mouse body mass between treatment groups (data not shown). All therapies produced a significant tumor growth delay. The levels of bone marrow–derived circulating endothelial progenitor (CEP) cells in the peripheral blood were measured by four-color flow-cytometric analysis of blood collected following the treatment period (Fig. 3). We have previously shown that suppression of peripheral blood CEP levels can be used as a surrogate marker for the efficacy of antiangiogenic therapy (17). Based on this assay, we find that all therapies tested except for MTD cyclophosphamide, as expected, have an antiangiogenic effect.

The TSLC fraction in treated tumors was assessed by primary tumor sphere-forming assay of enzymatically dissociated tumors harvested at the end of the treatment period (Fig. 4*A* and *B*). MTD cyclophosphamide or DC101 had no effect on the fraction of SFU within the tumor, whereas all other therapies significantly reduced the SFU fraction (Fig. 4*A*). Several therapies caused a small but significant increase in the size of primary tumor spheres, with MTD cyclophosphamide producing the greatest increase in sphere size (Fig. 4*B*). Furthermore, MTD cyclophosphamide was the only treatment to cause an increase in sphere size without a concomitant decrease in SFU fraction. Primary tumor spheres were dissociated, and secondary sphere-forming assays were done (Fig. 4*C* and *D*). Sphere-forming capacity was increased in the secondary compared with the primary assay across all groups, suggesting that SFU in the tumor are capable of self-renewal and

expand in the culture conditions used for the assay. Intergroup relationships of sphere-forming capacity observed in the primary assay were generally maintained in the secondary assay, indicating that certain therapies preferentially impact self-renewing tumor cells.

MTD cyclophosphamide is generally considered to work primarily via direct tumor cell cytotoxicity. Indeed, we find that MTD cyclophosphamide results in a significant tumor growth delay (Fig. 2) in the absence of any antiangiogenic effects as assessed by the peripheral blood CEP assay (Fig. 3). DC101 is a rat monoclonal antibody that blocks VEGF binding to the mouse VEGFR2 and, therefore, produces a tumor growth delay strictly by inhibition of angiogenesis. Our results with MTD cyclophosphamide and DC101 therefore indicate that neither inhibition of angiogenesis with a targeted biological agent alone nor tumor cell cytotoxicity alone is sufficient to reduce the TSLC fraction in the tumor. Although we did not see an increase in SFU following MTD cyclophosphamide, an outcome that has been predicted for tumors treated with cytotoxic chemotherapy (6), the increased sphere diameter resulting from MTD cyclophosphamide in the primary and secondary assays may reflect selection for self-renewing tumor cells with the highest proliferative capacity.

Although LDM cyclophosphamide was not as strongly antiangiogenic as DC101 (Fig. 3), both therapies produced a comparable tumor growth delay (Fig. 2). This suggests that another factor unrelated to the inhibition of angiogenesis contributes to the efficacy of LDM cyclophosphamide. Given the mechanism of action of cyclophosphamide (a DNA alkylating agent), the most plausible explanation is that LDM cyclophosphamide, in addition to its antiangiogenic effects, produces some tumor cell cytotoxic effects as well in C6 xenograft tumors. Therefore, all treatment groups that cause a reduction in tumor TSLC fraction, namely, LDM cyclophosphamide, LDM cyclophosphamide + DC101, MTD cyclophosphamide + DC101, and bolus dose + LDM cyclophosphamide, produce both antiangiogenic and tumor cell cytotoxic effects. Notably, the addition of DC101 enhanced the TSLC-reducing effects of LDM cyclophosphamide and conferred TSLC-reducing ability to MTD cyclophosphamide, demonstrating that angiogenesis inhibition plays a significant role in the observed reductions in the TSLC fraction. The addition of a bolus

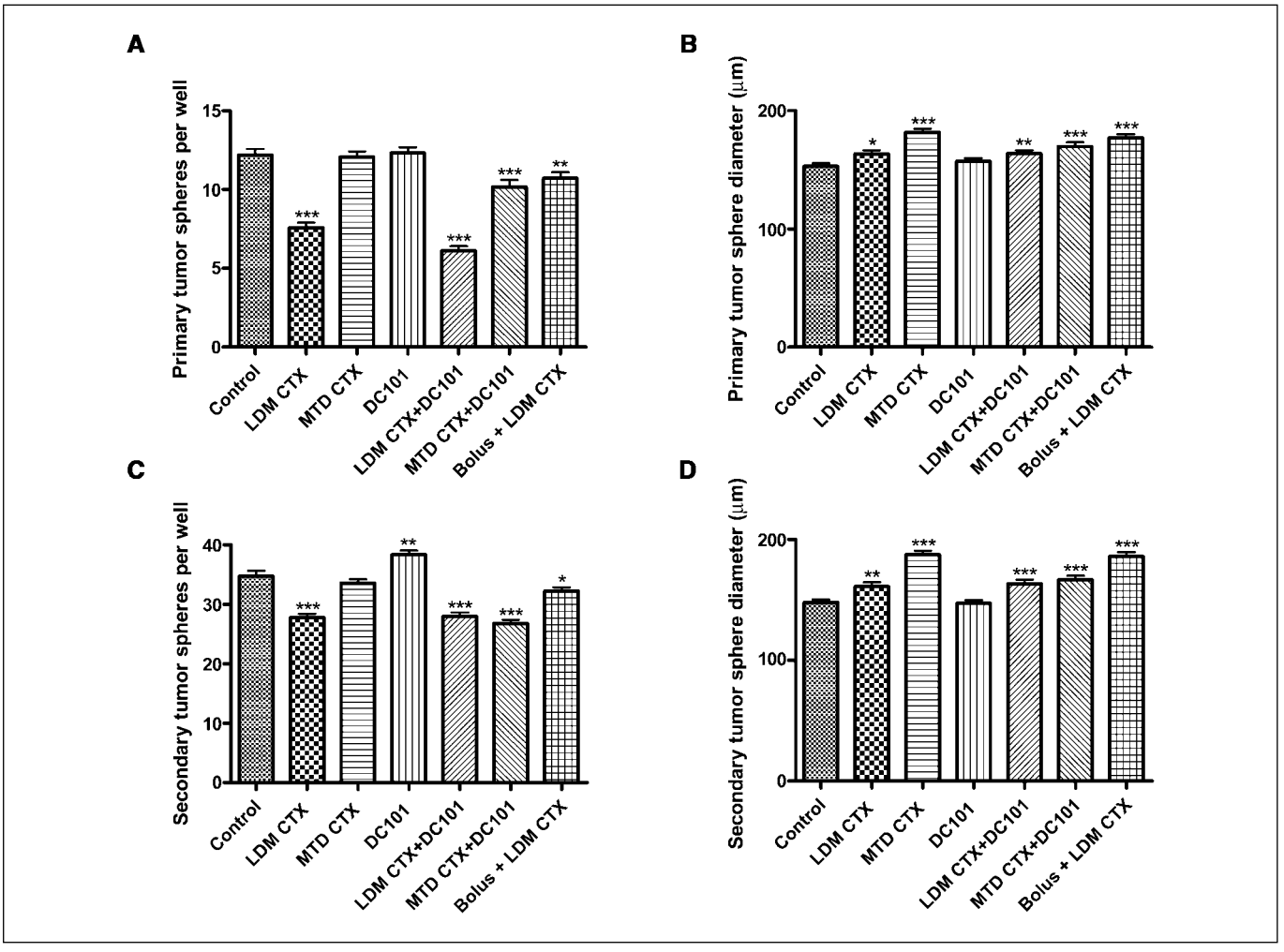

Figure 4. Primary and secondary sphere-forming assays of C6 tumors from mice following treatment. Following 2 wks of treatment with a variety of therapies (see Fig. 2), tumors were excised, digested, and subjected to primary (*A* and *B*) and subsequent secondary (*C* and *D*) sphere-forming assays as described in Materials and Methods. Each tumor was assayed individually, and data were pooled for each treatment group. Results represented as mean ± SEM. *A,* ***, $P = 0.0008$ (MTD cyclophosphamide + DC101); **, $P = 0.009$; ***, $P < 0.0001$ versus control (Student's *t* test). *B,* *, $P = 0.0102$; **, $P = 0.0072$; ***, $P = 0.0002$ (MTD cyclophosphamide + DC101); ***, $P < 0.0001$ versus control (Student's *t* test). *C,* **, $P = 0.0022$; *, $P = 0.0288$; ***, $P < 0.0001$ versus control (Student's *t* test). *D,* **, $P = 0.0025$; ***, $P = 0.0004$ (LDM cyclophosphamide + DC101); ***, $P < 0.0001$ versus control (Student's *t* test).

cyclophosphamide dose lessened the TSLC-reducing capacity of LDM cyclophosphamide, and the combination of LDM cyclophosphamide + DC101 caused the most significant reduction in TSLC. Taken together, these observations suggest that strong inhibition of angiogenesis, in combination with frequent low-level cytotoxic effects to tumor cells (as opposed to the strong cytotoxic effects resulting from bolus cyclophosphamide doses), is optimal for selective reduction of the TSLC fraction.

Discussion

A number of studies have shown that targeted antiangiogenic agents and chemotherapy often have additive or synergistic effects when used in combination. Indeed, in the clinic, antiangiogenic drugs used as monotherapies have little, if any, discernible therapeutic (survival) benefit in the treatment of advanced-stage cancers and must be combined with chemotherapy to show such a benefit (16). Several models have been proposed to explain the apparent chemosensitizing effects of antiangiogenic therapy (16). These models include "vessel normalization," resulting in transient drops in interstitial fluid pressure, increased blood flow, and reduced hypoxia within tumors (18), slowing down tumor cell repopulation between successive cycles of chemotherapy (19) and facilitating the targeting of dividing endothelial cells in the tumor vasculature and/or bone marrow–derived CEP cells (13–17). Our current results suggest yet another possible way in which antiangiogenic therapy and cytotoxic chemotherapy may work together in some cases, namely, by eliciting preferential eradication of the TSLC fraction, as in our glioma model. Our results show that whereas neither antiangiogenic nor tumor cell cytotoxic effects alone are sufficient to reduce the TSLC fraction, therapies combining these effects are capable of selectively eliminating TSLC from the tumor. One possible explanation for these observations is that antiangiogenic therapies disrupt a glioma TSLC vascular niche. The subsequent loss in communication between TSLC and their niche would elicit a reduction or loss of certain stem cell characteristics, which could include aspects of stem cell–associated drug resistance, and may result in, for example, increased proliferation rate and reduced DNA repair capacity in TSLC. Such changes would sensitize the TSLC to the cytotoxic effects of cyclophosphamide, thereby allowing combinatorial therapies to selectively eliminate TSLC. This hypothesis represents a novel model by which antiangiogenic therapy may sensitize tumors to the effects of chemotherapy, providing further rationale for the combination of these therapies in clinical practice. It may also help explain why an antiangiogenic drug such as bevacizumab may enhance the efficacy of chemotherapy for prolonging progression-free survival and overall survival in the absence of a major increase in the tumor response rate (i.e., rapid and significant tumor shrinkages; ref. 20) because the TSLC population represents only a minor fraction of the overall tumor mass. Furthermore, our data suggest that bolus cytotoxic doses of chemotherapy may select for highly proliferative self-renewing tumor cells, whereas, in contrast, frequent, low-dose schedules of the same agent eliminate these cells, indicating that LDM schedules of chemotherapy may be superior to MTD schedules as the partner therapy to an antiangiogenic agent and for targeting the TSLC population.

In addition to demonstrating a novel function of antiangiogenic therapy, our data provide new insight into TSLC biology. Specifically, we show that factors secreted by endothelial cells increase the TSLC fraction in C6 cultures, and that inhibition of angiogenesis plays a key role in the elimination of TSLC by antiangiogenic plus cytotoxic therapy. These findings, consistent with another recently published study (10), suggest that the vascular microenvironment has a role in maintaining brain TSLC identity and function. Furthermore, our work indicates that targeting of a TSLC supportive niche microenvironment may be a feasible strategy (or component of a strategy) for targeted eradication of TSLC.

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