Soluble CD70: a novel immunotherapeutic agent for experimental glioblastoma

Laboratory investigation

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Object. Given the overall poor outcome with current treatment strategies in malignant gliomas, immunotherapy has been considered a promising experimental approach to glioblastoma for more than 2 decades. A cell surface molecule, CD70, may induce potent antitumor immune responses via activation of the costimulatory receptor CD27 expressed on immune effector cells. There is evidence that a soluble form of CD70 (sCD70) may exhibit biological activity, too. A soluble costimulatory ligand is attractive because it may facilitate immune activation and may achieve a superior tissue distribution.

Methods. To test the antiglioma effect of sCD70, the authors genetically modified SMA-560 mouse glioma cells to secrete the extracellular domain of CD70. They assessed the immunogenicity of the transfected cells in cocultures with immune effector cells by the determination of immune cell proliferation and the release of interferon-γ. Syngeneic VM/Dk mice were implanted orthotopically with control or sCD70-releasing glioma cells to determine a survival benefit mediated by sCD70. Depletion studies were performed to identify the cellular mediators of prolonged survival of sCD70-releasing glioma-bearing mice.

Results. The authors found that ectopic expression of sCD70 enhanced the proliferation and interferon-γ release of syngeneic splenocytes in vitro. More importantly, sCD70 prolonged the survival of syngeneic VM/Dk mice bearing intracranial SMA-560 gliomas. The survival rate at 60 days increased from 5 to 45%. Antibody-mediated depletion of CD8-positive T cells abrogates the survival advantage conferred by sCD70.

Conclusions. These data suggest that sCD70 is a potent stimulator of antiglioma immune responses that depend critically on CD8-positive T cells. Soluble CD70 could be a powerful adjuvant for future immunotherapy trials for glioblastoma. (DOI: 10.3171/2009.11 JNS09901)

Key Words • soluble CD70 • glioma • immunotherapy • SMA-560 • T cell

Abbreviations used in this paper: ELISA = enzyme-linked immunosorbent assay; FCS = fetal calf serum; IFN = interferon; NK = natural killer; sCD70 = soluble CD70; TGF = transforming growth factor.

Glioblastoma is the most common of primary brain tumors. Despite recent advances, the overall limited median survival of < 12 months has not been significantly improved in recent decades.22 This has led to growing interest in developing alternative therapies, including immunological strategies.6,15,25 A broad set of cells of the immune system can be found in gliomas in vivo, including cytotoxic T or NK cells.11 However, a local immunosuppressive micro-milieu inhibits an efficient antglioma immune response, with the immunosuppressive cytokine TGF-β playing a key role.23 Counteracting immunosuppression, together with harnessing the effector function of tumor-infiltrating immune cells, might improve the efficacy of immunotherapeutic strategies for glioblastoma in the future.

The 2-signal model proposes that T-cell activation requires specific antigen recognition via the T-cell receptor while the second signal is provided by a costimulatory molecule such as CD28 and its ligands CD80/CD86.10 Without a costimulatory signal, T cells become “anergized,” and this absence of costimulation is one of many ways in which tumor cells evade the host’s immune system.12 Both CD27 and other members of the tumor necrosis factor receptor superfamily compose another group of costimulatory molecules.21 The CD27 is expressed on naïve CD4-positive and CD8-positive T, B, and subsets of NK cells.18,20 Its ligand, CD70, is a Type II transmembrane protein of the tumor necrosis factor superfamily.5 Expression of CD70 is mainly limited to activated T and B cells and mature dendritic cells.3,18 Interactions of CD27/CD70 promote the accumulation of
cytotoxic effector cells, T-cell memory, expansion of B-cell populations, and cytotoxic function in NK cells, and also facilitate antitumor immune responses.\textsuperscript{1,4,9,10,17} Human CD70 molecules are active in murine models and vice versa. Therefore, species specificity is not observed on a functional level.\textsuperscript{3}

Expression of CD70 has been demonstrated in glioma cell lines and in human gliomas in vivo.\textsuperscript{2,24} The CD70/CD27 interactions in the glioma context have yielded conflicting results in that CD70 expressed on glioma cells triggered immune activation or apoptosis in in vitro paradigms.\textsuperscript{2,24} In contrast, in vivo there is a potent immune stimulation in mouse glioma models that overrides any possible immune inhibitory effects.\textsuperscript{2}

Here, we studied the possible therapeutic value of sCD70 in a syngeneic mouse glioma model. The use of a soluble immune stimulatory agent would have major advantages compared with the necessity of enforced membrane CD70 expression in established gliomas in a clinical setting.

Methods

Cell Lines and Reagents

Murine SMA-560 glioma cells, kindly provided by D.D. Bigner (Durham, NC), were grown in Dulbecco modified Eagle medium (Gibco Life Technologies) supplemented with 10% heat-inactivated FCS (Biochrom KG), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco). The generation of cell surface CD70-expressing glioma cells has been previously described.\textsuperscript{2} To generate the sCD70-secreting glioma cell line SMA-560 sCD70, the retroviral plasmid DFG containing cDNA for sCD70, kindly provided by C. Cormary (Toulouse, France), was transfected into SMA-560 cells using Metafectene (Biontex).\textsuperscript{3}

Growth Assay

Either SMA-560 mock or sCD70 cells were seeded in 96-well plates in triplicate. Cell density of the attached cells was assessed by crystal violet staining after 24, 48, 72, or 96 hours. Briefly, the cell culture medium was removed, and surviving cells were stained with 0.5% crystal violet in 20% methanol for 20 minutes at room temperature. The plates were washed extensively under running tap water and air dried, and optical density values were read in an ELISA reader at a wavelength of 550 nm.

Immunoblot

The general procedure has been previously described.\textsuperscript{24} After 24 hours of incubation in serum-free medium, cellular supernatants were collected and concentrated in a Vivaspin 15 centrifugal filter device (5000 MWCO, Sartorius). Recombinant sCD70 (Axxora) was used as the positive control (100 ng/lane). Anti-human CD70 (clone C-20) was obtained from Santa Cruz Biotechnology.

Flow Cytometry

Adherent glioma cells were detached using Accutase (Millipore) and blocked with 2% FCS in phosphate-buffered saline. The cells were incubated for 30 minutes on ice using the antibody of interest: anti-human CD70 (clone K1-24), anti-mouse CD70 (clone FR70), anti-mouse CD8a (clone 53–6.7), or anti-mouse NK1.1 (clone PK136) (BD Biosciences). Flow cytometry was performed with a Dako flow cytometer. Signal intensity was calculated by dividing the mean fluorescence obtained with the specific antibody divided by signal intensity obtained with the isotype control antibody (specific fluorescence index).

Animal Experiments

The VM/Dk mice were obtained from the TSE Resource Center (Berkshire, UK). Five × 10\textsuperscript{3} SMA-560 cells were injected into the right striatum.\textsuperscript{2} The mice were killed at the onset of neurological symptoms. For depletion experiments anti-NK1.1 (clone PK136 [200 μg]) or anti-CD8 (clone 2.43 [20 μg]) antibodies were used. The depleting antibodies were given 2 days before and on the day of tumor inoculation. Further depletion and confirmation by flow cytometry were performed at weekly intervals. Animal experiments were carried out according to German animal protection laws and to the standards in the Guide for the Care and Use of Laboratory Animals and approved by the local authorities.

Splenocyte Proliferation

Splenocytes obtained in naïve VM/Dk mice were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Splenocytes (5 × 10\textsuperscript{5} cells) were then stimulated by coculturing with 5 × 10\textsuperscript{5} irradiated (20 Gy) SMA-560 sCD70 or mock cells for 72 hours. The cells were pulsed for 24 hours with 1-μCi/well [methyl-\textsuperscript{3}H]thymidine (Amersham Life Science) and assayed for thymidine incorporation using a 1450 Microbeta Plus liquid scintillation counter (Wallac). For blocking experiments, the anti-CD70 antibody (clone FR70) was used (eBioscience).

Enzyme-Linked Immunosorbent Assay

Supernatants from splenocytes cocultured with either SMA-560 sCD70 or mock cells were harvested. Concentrations of IFN-γ (PeproTech) were determined by sandwich ELISA.

Immunohistochemistry

Brain cryosections were prepared from mice killed on Day 10 following intracranial SMA-560 glioma cell inoculation. Sections were fixed in 4% formalin for CD8 staining or with acetone for CD4, Ly49G2 (NK cell), and CD11b staining. Blocking was done with 10% normal rabbit serum for all antibodies, except for Ly49G2 with 2% normal rabbit serum and 2% bovine serum albumin. The sections were stained with antibodies (Pharmingen) to CD8 (clone 53–6.7; 1:50 dilution), CD4 (clone RM4–5), or CD11b (clone M1/70). The antibody Ly49G2 (clone 4D11; 1:25 dilution) was used to detect NK cells. A biotinylated anti–rat secondary antibody (BA-4001, Vector) was used at 1:100 dilution. Avidin conjugate (ABC Kit,
Vector) was added and developed with diaminobenzidine. Mouse thymus (CD4 and CD8) and spleen (Ly49G2 and CD11b) served as positive controls.

**Statistical Analysis**

Analysis of statistical significance was performed using a 2-tailed Student t-test and the log-rank test for survival analysis. GraphPad Prism 5 software was used for calculations.

**Results**

**Generation of SMA-560 Cells Releasing sCD70**

The spontaneous murine astrocytoma cell line SMA-560 is syngeneic in VM/Dk mice and does not constitutively express membranous or sCD70 (Fig. 1A). Either DFG-sCD70 or mock plasmid was stably transfected into SMA-560 cells. The SMA-560 mock and sCD70-transfected cells displayed equivalent proliferation (Fig. 1B).

**Fig. 1.** Expression of sCD70 in SMA-560 murine glioma cells. A: Membranous expression of CD70 on SMA-560 mock and sCD70 cells was assessed using flow cytometry. The filled profile indicates the specific antibody, and the open profile indicates the isotype antibody. (SFI of SMA-560 mock, 1.07; that of sCD70, 1.00; 1 representative experiment is shown; 3 were performed.) B: Proliferation of SMA-560 mock and sCD70 cells was measured in a crystal violet assay. Eight thousand cells per well were seeded in 96-well plates in triplicate. Cells were stained with crystal violet after 24, 48, 72, and 96 hours (1 representative experiment is shown; 3 were performed). C: The levels of sCD70 were assessed by immunoblot in the supernatant of SMA-560 mock or sCD70 transfectants directly or after in vivo passaging for 30 days. Recombinant sCD70 (10 ng) was used as a positive control.

**Fig. 2.** Functional activity of sCD70 in SMA-560 murine glioma cells. A: The SMA-560 sCD70 or mock transfectants were irradiated with 20 Gy, and 10^3 cells were cocultured with 10^6 splenocytes per well for 72 hours. [Methyl-3H]-thymidine (1 µCi/well) was added for 24 hours and splenocyte proliferation was quantified by liquid scintillation counting. An sCD70 blocking antibody was added as a control where indicated (2 µg/ml). *p < 0.05, **p < 0.01 (Student 2 tailed t-test). B: Interferon-gamma was measured in cell culture supernatants after a 72-hour coculture of splenocytes with irradiated (20 Gy) SMA-560 glioma transfectants by ELISA. An sCD70 blocking antibody was added as a control where indicated (2 µg/ml). *p < 0.01, ***p < 0.001 (Student 2 tailed t-test).
The release of sCD70 into the cell culture supernatant was ascertained by immunoblot (Fig. 1C). The recombinant sCD70 is fused to a linker peptide and a FLAG-tag, which explains the slight increase in size. Transgene expression was stable as demonstrated by the continuous release of sCD70 from cells explanted from a VM/Dk mouse 30 days after intracranial implantation (Fig. 1C, Lane 4).

**Soluble CD70–Dependent Stimulation and Activation of Splenocytes In Vitro**

Splenocytes from VM/Dk mice were cocultured with SMA-560 sCD70 or mock-transfected cells to determine whether sCD70 was functionally active in eliciting immune activation. Irradiated sCD70- or mock-transfected glioma cells cultured without splenocytes demonstrated very little proliferation (data not shown). The splenocytes incubated with sCD70-secreting SMA-560 cells were highly proliferative. The proliferative activity was sCD70 dependent, as confirmed by the addition of sCD70 blocking antibody (Fig. 2A). The activation of splenocytes in vitro was studied in cocultures of $5 \times 10^6$ VM/Dk splenocytes and either $5 \times 10^4$ irradiated SMA-560 mock or sCD70 cells. Interferon-gamma was measured in the supernatant by ELISA. The levels of IFN-γ released were significantly elevated in the supernatant from splenocytes cocultured with the sCD70-releasing glioma cells. Enhanced IFN-γ production was reverted by a blocking anti-sCD70 antibody (Fig. 2B).

**Survival Advantage Provided by sCD70 in Intracranial Tumor–Bearing Mice**

To assess the ability of sCD70 to modulate orthotopic glioma growth in vivo, $5 \times 10^3$ SMA-560 sCD70 or mock-transfected cells were stereotactically implanted into the right striatum of VM/Dk mice. All animals except 2 in the control group displayed neurological symptoms and had to be killed by Day 25. Forty-five percent (9) of the mice injected with sCD70-secreting SMA-560 cells survived for more than 60 days, compared with 5% (1 mouse) in the control group (Fig. 3A). Three of the surviving animals from the sCD70-secreting group were available for a re-challenge with SMA-560 wild-type cells in the contralateral hemisphere more than 90 days after first implantation. These mice exhibited a prolonged survival compared with the control group at first challenge (median survival 20 days). One mouse died after 33 days, 1 after 55 days, and 1 survived more than 60 days, indicating the generation of an immunological memory following rejection of sCD70-expressing SMA-560 cells.

**Soluble CD70 Promotes Immune Cell Infiltration in the SMA-560 Syngeneic Mouse Glioma Model**

Some animals in each group were killed on Day 10 after glioma cell implantation for immunohistochemical analysis. The sCD70-secreting tumors demonstrated infiltration of CD8-positive cells into the periphery of the tumor and in the perivascular regions, whereas CD8-positive T cells were virtually absent in the control tumors. In contrast, both sCD70-secreting and mock tumors ex-

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**Fig. 3.** Soluble CD70 was shown to promote survival of intracranial glioma-bearing mice. A: Five $10^3$ SMA-560 sCD70 or mock transfectants were inoculated intracerebrally into VM/Dk mice. Survival data for 20 mice from the SMA-560 sCD70 group and 19 mice from the SMA-560 mock group are presented as a Kaplan-Meier survival plot. Survival data from 2 independent experiments with similar results were pooled ($p < 0.01$, log-rank test). B: Either SMA-560 sCD70 or SMA-560 mock cells were implanted into the right striatum of VM/Dk mice. The mice were sacrificed on Day 10 and H & E staining was performed to identify the inoculated tumor (arrows in the sCD70 group point to the margin of the tumor). Expression of CD8 was assessed by immunohistochemistry using anti-CD8 antibody, CD4 expression using anti-CD4, and macrophage infiltration using anti-CD11b (arrows point to examples of stained cells). Bar = 40 µm.
Soluble CD70 Immune Activation Mediated by CD8-Positive T Cells

To explore the role of immune effector cells in mediating survival, in vivo depletion of CD8-positive T or NK1.1-positive cells was performed. The depletion of CD8-positive or NK cells was verified by flow cytometry (Fig. 4A). The survival advantage conferred by sCD70 was abolished when CD8-positive cells were depleted (Fig. 4B). In contrast, the depletion of NK cells did not influence survival (Fig. 4C).

Discussion

Immunotherapeutic approaches to glioblastoma have not resulted in a major breakthrough despite considerable efforts both in the laboratory and in the clinic. The most advanced approach involves vaccination in patients with glioblastomas expressing the VIII mutant of the epidermal growth factor receptor.14 The lack of efficacy of immunotherapy in gliomas may be attributed to the release by glioblastoma cells of various immunosuppressive molecules, notably TGF-β. Accordingly, we have been interested in identifying immune stimulatory molecules that could be used to alter immune responsiveness in the glioma context and to possibly allow the generation of relevant immune responses to glioma cells. In that regard, we have previously reported that CD70 is aberrantly expressed by glioma cells and mediates both immune inhibitory and immune stimulatory effects in vitro.24 Immune stimulatory effects were strongly dominant in rodent glioma models in vivo.2 The application of a soluble variant of CD70 would greatly facilitate the clinical application of this approach because this molecule could be administered locally during surgery, systemically, or both, but gene therapy strategies to transduce glioma cells to release CD70 would not be necessary. Immune-activating molecules such as NKG2D ligands, however, are strongly immune stimulating when expressed at the cell surface, but they act as immune inhibitory molecules in their soluble form.7,8,13 In the present study we found that SMA-560 mouse glioma cells engineered to secrete sCD70 enhance the proliferation of syngeneic splenocytes and their secretion of IFN-γ. In syngeneic VM/Dk mice, the local release of sCD70 leads to an enhanced infiltration of CD8-positive T cells into orthotopic gliomas. This is consistent with an enhanced infiltration of CD8-positive T cells into subcutaneous SMA-560 gliomas expressing membrane-bound CD70.2 The survival advantage for the sCD70-secreting tumors is abolished when CD8-positive T cells are depleted. In contrast, the depletion of NK1.1-positive cells does not influence the survival of the animals, corresponding to what has been described previously in a murine mammary adenocarcinoma model.1 This suggests an antiglioma immune-stimulating action of sCD70, with CD8-positive T cells playing a decisive role in this syngeneic orthotopic mouse glioma model in vivo.

Compared with the present study, the forced expression of membrane-bound CD70 has exhibited a superior survival effect.2 However, the latter approach would be impeded by technical drawbacks in a clinical situation. Soluble CD70 is much more attractive because it would bypass problems of administration into the glioma and theoretically be more widely distributed throughout the tumor and surrounding tissues. Interestingly, the survival of mice expressing sCD70 was rather mixed, with some animals apparently deriving little benefit and some animals having prolonged survival (Fig. 5A). These data sug-
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gest a dosing or threshold effect for sCD70 that would have to be reached in a clinical setting, too. A similarly heterogeneous response has been observed in another immunotherapy approach in the same syngeneic mouse glioma model that was based on the pharmacological neutralization of TGF-β.39

Conclusions

Soluble CD70 is shown here to be a potent stimulator of antiglioma immune responses in the syngeneic SMA-560/VM/Dk mouse glioma model. The immune stimulation exerted by sCD70 depends critically on CD8-positive T cells. Soluble CD70 could be a powerful adjuvant for future immunotherapy trials for glioblastoma.

Disclosure

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