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Report

Inhibition of intracranial glioma growth by endometrial regenerative cells

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Animal studies have demonstrated that selective tropism of mesenchymal stem cells (MSC) for glioma may be used as a means of selective delivery of cytotoxic payloads. Endometrial Regenerative Cells (ERC) are a population of mesenchymal-like cells which possesse pluripotent differentiation capacity and is characterized by unique surface markers and growth factor production. In this study we sought to determine whether unmanipulated ERC would alter the growth of glioma using the aggressive C6/LacZ7 (C6) into Sprague Dawley rat model. ERC administration by intravenous (i.v.) or intratumoral (i.t.) showed significant inhibition of glioma: volume reduction of 49% after i.v. treatment ($p < 0.05$), and about 46% i.t. treatment ($p < 0.05$). Tumor reduction was associated with inhibition of angiogenesis and reduced numbers of CD133 positive cells in the incranial tumor. Despite the angiogenic potential of ERC in the hindlimb ischemia model, these data support a paradoxical tumor inhibitory activity of ERC. Further studies are needed to determine the qualitative differences between physiological angiogenesis, which seems to be supported by ERC and tumor angiogenesis which appeared to be inhibited.

Introduction

A number of studies support the notion that stem cell/progenitor cell administration is a potential way of suppressing tumor growth. Aboody et al. reported that subsequent to implantation of fetal-derived neural stem cells (NSCs) into experimental intracranial glioma in adult rodents, the cells preferentially distribute throughout the tumor while not integrating into non-malignant tissue.[1] This tropism of NSCs for glioma was used by others to as a delivery means of therapeutic genes to tumors.[2,3] Other studies have demonstrated that mesenchymal stem cells (MSCs) can also selectively integrate into gliomas after intravascular or local delivery.[4] Human skin-derived progenitor cells have also demonstrated selective trophism for malignant tissue, and more interestingly, had the ability to inhibit tumor growth in an unmanipulated manner.[5]

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Endometrial regenerative cells (ERCs) are a novel stem cell population derived from menstrual blood expressing some but not all MSC markers, while lacking hematopoietic stem cell markers.[6] ERC have been demonstrated to possess a degree of pluripotency, as well as express the embryonic stem cell marker Oct-4. In agreement with the notion that these cells are involved in the cyclical stimulation of endometrial angiogenesis, we previously reported ERC are potently proangiogenic in vitro and in vivo,[7] which is supported by their high expression of MMP3 and MMP10.[6] Previous studies have shown bone marrow derived cells, not exclusively endothelial precursors, but also monocytes and MSC, when administered to tumor bearing mice augment tumor angiogenesis and progression.[8-10] Given that we are exploring the possibility of clinical translation of ERC, we sought to examine whether administration of these cells would affect growth of the aggressive C6/LacZ7 (C6) glioma tumor in rats. We found an inhibitory effect on tumor growth, accompanied by reduction in angiogenesis and numbers of CD133 positive tumor cells.

Results

ERC administration inhibits C6 tumor growth. In order to assess effects of ERC in an in vivo tumor model, 1 x 10^6 C6 cells were implanted intracranially on day zero in the right frontal lobe of Sprague Dawley rats. On day 2, ERC were administered intravenously (i.v.) or intratumorally (i.t.) at a concentration of 3 x 10^6 or 1 x 10^6 cells per animal, respectively. Control animals were left untreated. Injections were uneventful with no observation of procedure associated adverse reactions. All animals were sacrificed on day 14. Tumor measurements were made based on a series of frozen sections and stained for B-gal expression (Fig. 1). A reduction of about 49% in overall tumor volume was observed after i.v ERC treatment ($p < 0.05$) and about 46% in animals receiving ERC i.t. ($p < 0.05$).

ERC administration associated with reduced neovascularization. Tumor blood vessels density was detected by counting of CD34 positive cells having endothelial morphology. As seen in Figure 2, lower numbers of blood vessels were observed in tumors from animals treated with ERC i.v. and i.t., as compared to controls. Specifically, we found an approximate 50% reduction in blood vessel density in i.v. treatment group (control group vs i.v group : 72 ± 18 vs. 35 ± 11, $p < 0.001$) and approximately 37% reduction in the i.t. treated group (42 ± 9, $p < 0.001$).

Reduction of CD133 positive C6 Cells in ERC treated animals. Tracking of putative glioma stem cells was feasible because of coexpression of CD133 on B-gal expressing cells. We identified a reduction of approximately 67% and 33% in the number of CD133 positive cells in rats glioma tissues treated with ERC i.v. (9 ± 7, $p < 0.01$) and i.t.(18 ± 5, $p < 0.05$) respectively as compared to control group (27 ± 10).

Discussion

We have previously reported that ERC are a population of endometrial derived stem cells having ability to differentiate into numerous non-hematopoietic tissues.[6] Given the ease of collection, ability for large scale expansion, and lack of need for tissue matching to achieve therapeutic effects, a clinical translation program was initiated to the goal of developing an "off the shelf therapy" for critical limb ischemia (CLI). As part of any such endeavor, it was critical to elucidate not only whether ERC can themselves transform into tumor tissue, which we published previously is not the case,[7] but also whether they support the growth of existing tumors. This was a particular concern because of the potent angiogenic activities of ERC in hindlimb ischemia models. We have previously reported that ERC administration did not accelerate tumor growth in a UVB induced model of skin cancer,[7] and therefore we sought to extend these studies into a model of possible therapeutic relevance.

The intrinsic affinity of various progenitor cells to tumors has conventionally been explained as a result of injury-based chemoattraction.[11,12] In the similar manner to which bone marrow progenitors mobilize to injured myocardium after an infarct,[13,14] or to injured brain tissue after a stroke,[15,16] it is believed that endogenous stem cells are attracted by tumor induced tissue injury. Numerous factors secreted by tumors or adjacent tissue including SDF-1,[8] tissue factor[17] and inflammatory mediators,[18] can act as stem cell chemoattractants. For this reason, various groups have used neural progenitors, or other types of stem cells as vectors for delivery of therapeutic genes or products thereof. While the notion of using stem cells to target tumors is relatively accepted, a pressing question is whether unmanipulated stem cells inhibit or augment tumor progression.

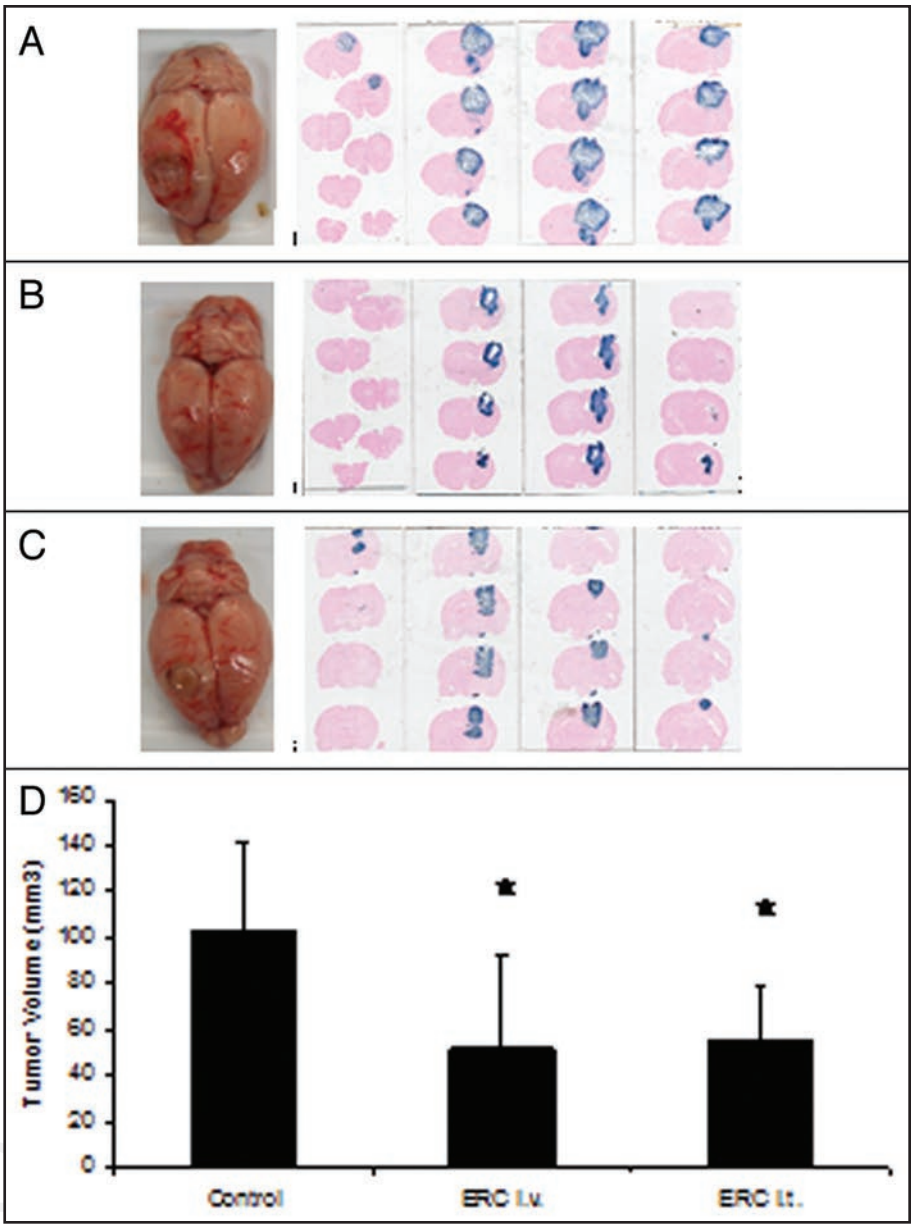

Figure 1. ERC Administration Inhibits C6 Tumors In Vivo. Sprague Dawley rats were implanted with 1 million C6 tumor cells in the right frontal lobe and divided into three groups: Group (1) untreated controls (A); Group (2) i.v. administration of 3 million ERC on day 2 (B); and Group (3) 1 million ERC implanted locally at site of tumor implant on day 2 (C). Animals were sacrificed 14 days after tumor implantation and volume of X-gal positive tumors was quantified (D). Figures represent average of a total of 8 rats per group. *$p < 0.05$ according to t test.

Hypothetically, one would imagine that since stem cells secrete numerous growth factors and angiogenic factors, they would actually augment tumor growth. Conversely, given the natural tendency of numerous progenitor cells to differentiate, especially in the presence of inflammation,[19] it may be possible that administration of progenitor cells can directly induce tumor differentiation. This concept is supported by reports of melanoma differentiating into neurons and skin cells after implantation into fertilized chicken eggs.[20] Other studies have demonstrated that MSC directly secrete tumor inhibitory factors.[21,22] Before identification of MSC as a distinct cell type, reports exist of a bone marrow-derived non-cytotoxic tumor inhibitor of a low molecular weight, capable of inducing G_0 arrest/apoptosis of various tumor cells,[23,24] as well as inhibiting tumor growth in vivo.[25,26]

In our experiments we observed a profound inhibition of C6 glioma cells in animals treated with ERC either i.v. or i.t.. Suppression of tumor growth was not associated with necrosis but characterize by lower number of new blood vessels as identified morphologically and by anti-CD34 staining. Given that conditioned media of ERC cultures stimulates HUVEC proliferation in vitro,[7]

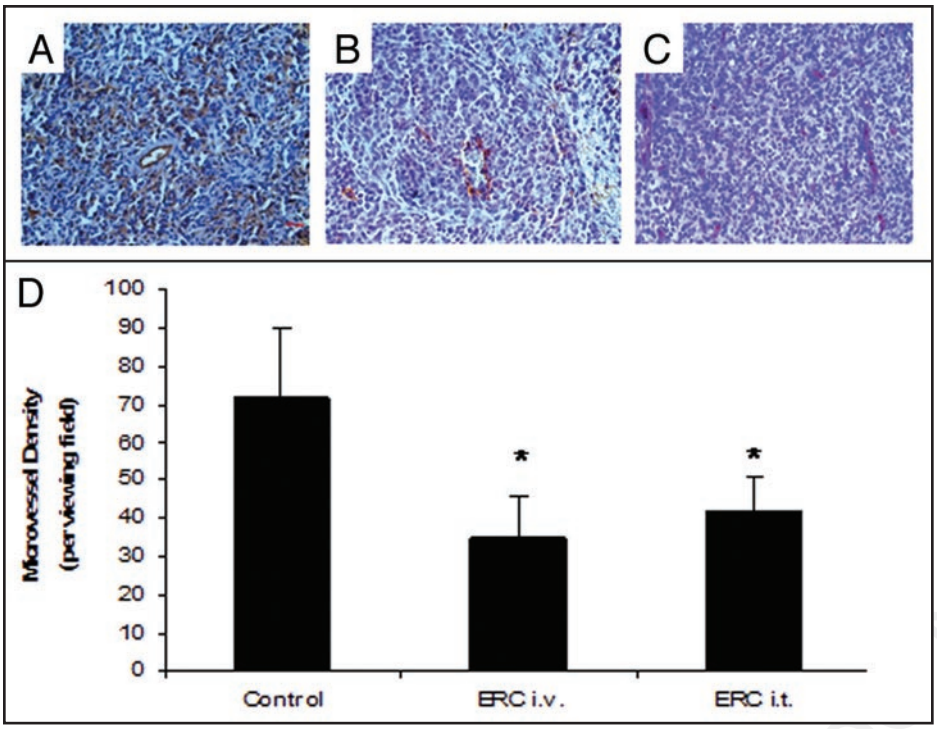

Figure 2. ERC Administration Associated with Reduced Tumor Angiogenesis. Tumor vascularization was determined by staining with anti-CD34. Tumors in mice of Group 1 (control) exhibited vessels with irregular diameter and tortuous morphology (A). Less vessels were observed in Group 2 (ERC i.v.) (B), as well as in Group 3 (ERC i.t.) (C). Vessel density was quantified by scanning the CD34-stained sections at low magnification (40x) to determine areas with the highest number of microvessels as hot spots. Microvessels were counted at a magnification of 200x in four hot spots on each section and microvessel density was calculated as the average per viewing field (D) *(t-test, $p < 0.001$).

we speculated that the ERC may be inhibiting tumor growth and as a result less angiogenesis was present. An alternative explanation may be that qualitative differences in angiogenesis between tumors and ischemic non-malignant tissues, such that ERC selectively stimulate physiological but not pathological angiogenesis. Previously it was published that induction of immunity to angiogenesis related molecules leads to a selective inhibition of tumor angiogenesis but not angiogenesis in wound healing or the corpus luteum.[27] Studies are currently underway to address these issues.

The inhibition of tumor growth could be associated with differentiation of tumor stem cells. Tumor stem cells are known to express CD133 and reside in hypoxic niches of tumors.[28,29] Others have demonstrated that MSC have preferential affinity towards hypoxic tissue.[30] Patel et al. reported on an ERC-like population expressing similar markers and originating from the endometrium.[31] His group demonstrated ERC-like cells express CXCR-4, the receptor for SDF-1, a factor secreted by hypoxic cells. Accordingly, it may be possible that the injected ERC were interacting/inhibition/differentiating CD133 tumor stem cells. While we observed reduction in these cells, further studies are required to identify the significance of this inhibition.

The possibility exists that induction of immunity to ERC-derived proteins may cross-react with tumor expressed antigens and account for reduction in tumor volume. Studies immunizing mice with human endothelial cells have demonstrated induction of antibodies to various integrins on the xenogeneic endothelium, which cross react with tumor-associated endothelium and mediate anticancer effects.[32,33] Since ERC express various angiogenic factors, it is possible that immunity was induced to factors such as PDFG-BB or MMPs, which blocked activity of the endogenous tumor secreted molecules. While we

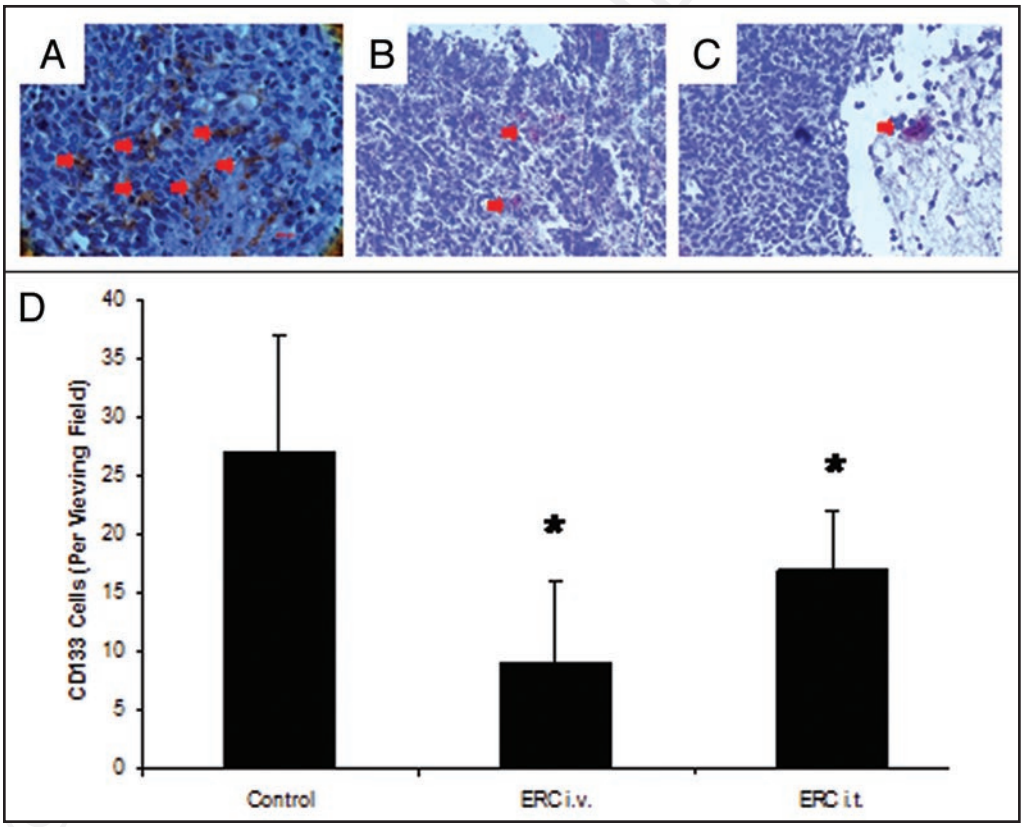

Figure 3. Reduction in CD133 Positive Glioma Cells after ERC Administration. Representative immunohistochemistry figures of glioma's from control (A), i.v. ERC injected (B), and i.t. ERC injected (C). Average CD133 positive cell per viewing field is depicted (D). a reduction of approximately 67% and 33% in the number of CD133 positive cells in rats glioma tissues treated with ERC i.v. and i.t., respectively. * t test $p < 0.05$.

AUTHOR: please refer to Figure 3 within the body text.

can not conclusively rule out this possible explanation, several lines are reasoning suggest it is not likely. Firstly, i.v. administration of antigens is not likely to induce immunological responses, but may even cause intravenous tolerance.[34] Secondly, inhibition of tumor growth was also observed by intratumoral injection of ERC, which was not likely to stimulate immune responses due to the local immune privilege associated with the CNS, as well as the tumor microenvironment.[35] Thirdly, we observed selective localization of labeled ERC associated with malignant tissue at the time of experiment termination (data not shown), thus making it unlikely that a potent anti-xenogeneic response was being mediated. Our previous study in the hindlimb ischemia model was conducted in immune competent BALB/c mice, and resulted in stimulation of angiogenesis despite a xenogeneic environment.[7]

In conclusion, we report that administration of ERC into a rat model of glioma seems to exert a therapeutic effect associated with inhibition of angiogenesis and reduction in tumor cells positive for the CD133 phenotype.

Materials and Methods

Cells. Menstrual blood was collected from a healthy female subject after menstrual blood flow initiated and ERC were cultured as previously described.[6] The cells were then subcultured and passaged twice a week. We collected 3 x 10^6 every time for intravenous injection and 1 x 10^6 for intra-tumor injection, cells were washed 2 times with sterile PBS to remove FPS and store in PBS for injection (group 2 and 3). C6/LacZ7 cells were purchased from ATCC (CRL-2303) and maintained according to the manufacturer's instructions. Cells were cultured in complete DMEM media with 10% Fetal bovine serum; 0.1 mM Non-Essential Amino Acids (NEAA), 1% penicillin/streptomycin, and 1% amphotericin B. Cells were incubated at 37°C in a fully humidified environment with 5% CO_2. An aliquot of cells were stained with X-gal during passaging to ensure β-gal expression.

Animal models. Male Sprague Dawley rats, age 50–80 days, weighing 250–400 g (Charles River, Wilmington, MA), were used for all experiments. Before tumor implantation, rats were anesthetized with ketamine hydrochloride/xylazine hydrochloride solution (Sigma). After rats were unconscious without pain reaction, they were placed in a surgical bed, with the head stabilized on the stereotactical frame (KOPF, Model 900 small Animal Stereotaxic Instrument), hair was shaved and cleaned with iodine solution, and then a sterile dress was used to cover the surgical area. The skin was incised to expose the skull. A 1mm diameter hole was drilled with a round diamond bur (1.0 mm, Stryler), with coordinates of AP +0.0; ML -2.5; DV -4.5 mm (right front lobe). A Hamilton Microliter Syringe was installed in a syringe holder with needle support (Model 1772-F) fixed on the KOPF stereotactical frame. 1 x 10^6 C6/Lacz7 cells suspended in a volume of 15 μl were injected slowly with a 26 gauge sterile needle.

Treatment groups. Rats were implanted intracranially with 1 x 10^6 C6 cells on day 0 and divided into 3 groups: Group A received no treatment; Group B was administered an intravenous injection of 3 x 10^6 stem cells in 1ml sterile PBS via the tail vein on day 2; and Group C was implanted sterotactically with 1 x 10^6 ERC in 10 μl sterile PBS in the same area as C6 implantation on day 2. All animals were sacrificed on day 14 and brain samples were collected and stored for frozen section and X-gal stain, or stored in formalin for paraffin section.

Frozen sectioning and X-gal stain. To evaluate the volumes of brain tumors, frozen brain samples were continuously cut into series coronary sections at a thickness of 20 μm in a Cryostat (Minotome *PLUS*™, TBS, NC). X-gal stain is as follows: Frozen sections were fixed with cold formalin (4°C) for 10 minutes. Wash slides with 3 changes of PBS for 5 minutes each and then rinse in distilled water. Then incubate slides in X-gal working solution at 37°C for 24 hours in humidified chamber, rinse sections in PBS for 2 x 5 minutes, counterstained with Vector® Nuclear Fast Red (Vector Lab) for 10 minutes, wash with water 5 minutes and then dehydrate with series alcohol and wash in Xylene, count with DPX.

Immunohistochemistry staining. Frozen sections were collected and thawed and air dry for 30 min, fixed in 100% acetone for 5 min in room temperature, rinse in PBS pH 7.4, 3 x 5 min, then sections were incubated in blocking solution (4% non-fat milk and 2% normal horse serum) for 60 min, then incubated with CD34 (C-18) (1:200, goat poly-IgG, Santa Cruz), CD133 (k-18) (1:200, goat polyclonal IgG, Santa Cruz) (diluted in 2% milk) overnight at 4°C. The second day, sections were rinsed in PBS and apply biotinylated horse anti-goat antibody (Vector Lab) at 1:200 for 2 hours and then 1% H_2O_2/PBS for 10 min, followed by the avidin-biotin complex (ABC) kit (Vector Laboratories) and visualized with diaminobenzidine (DAB). Sections were counterstained with hematoxylin, results were observed under the microscope and pictures stored in the computer for analysis. For paraffin sections, after deparaffinized and before blocking step, sections were putting in pressure cooker with Antigen Unmasking Solution (Vector H-3300, Vector lab) according to the manufacture's instructions. For assessment of microvessel density: CD34-stained sections were scanned at low magnification (40x) to determine areas with the highest number of microvessels as hot spots. Microvessels were counted at a magnification of 200x in 2 hot spots on each section and MVD was calculated as the average.

Statistical analysis. For comparing tumor volume and vessel density and CD133 positive cells in deferent group, data are present as means ± SD. Statistical analysis was carried out by the Student's t test. Probability (p) values < 0.05 are considered as significant.

Acknowledgements

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Conflicts of interest

Thomas E. Ichim and Neil H. Riordan are shareholders of Medistem Inc., (MEDS.OB).

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