The Influence of Retinoic Acid and Thalidomide on the Radiosensitivity of U343 Glioblastoma Cells

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Abstract. *Background/aim: 13-cis-retinoic acid (RA) and thalidomide have shown a synergistic anti-proliferative effect on U343 glioblastoma (GBM) cells. In the present study, we test if their combined treatment might enhance the radiosensitivity of these cells. Materials and Methods: The radiosensitivity of U343 GBM cells was determined by the colony formation assay. Fibroblast growth factor-2 (FGF2) gene expression was determined by a quantitative polymerase chain reaction (qPCR). Results: RA up-regulated FGF2 gene expression, which was abrogated by thalidomide. No radiosensitisation by RA was observed under standard culture conditions with 10% serum, but enhanced radiosensitivity was observed under 1% serum during irradiation. However, a synergistic effect with thalidomide was not observed. Conclusion: Growth factors in the culture medium may mask radiosensitization by RA while autocrine expression of FGF2 did not seem to be protective. Importantly, the anti-proliferative effect of RA in combination with thalidomide would not compromise the radiosensitivity of these GBM cells.*

Glioblastoma multiforme (GBM) is a common and aggressive primary brain tumor of glial origin (45). Despite the use of multi-modal treatment with surgery, radiotherapy (RT), and chemotherapy, the prognosis is poor with a median survival time of 14.6 months (41). The efficacy of RT is limited by the radioresistance of GBM (30). However, the molecular basis of this phenomenon is not well-understood.

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13-*cis*-retinoic acid (RA; isotretinoin) is a well-known differentiation-inducing agent (32) frequently used in clinical trials in patients with GBM (11). The most important mechanism of action of RA is transcriptional activation of nuclear retinoic acid receptors (RARs) (37). In GBM cells, this drug has been implicated in down-regulation of epidermal growth factor receptor (EGFR)-mediated signaling (39, 46), which may contribute to reduced sensitivity towards ionizing radiation (IR) (20). Some reports have suggested that 13-*cis* RA and *all*-trans-RA may increase radiosensitivity in different cancer cell lines (21, 43) but this effect was not pronounced. One possible explanation for the limited radiosensitizing *effect of* RA might be their induction of expression of homeobox (HOX) gene family members (32) such as HOXB7 (28).

The homeobox is an evolutionarily-conserved 180-base-pair sequence motif found in genes which play a crucial role in embryological development and differentiation (34). Homeoproteins encoded by HOX genes can bind to DNA, acting as transcription factors, and are frequently de-regulated in neoplastic disease (36). HOXB7 has an important role in carcinogenesis by stimulating tumour cell proliferation and angiogenesis (8, 10). Furthermore, HOXB7 may transactivate fibroblast growth factor 2 (*FGF2*) (9, 40) which can stimulate GBM growth (24) and protect cells from IR-induced injury (1). Thalidomide is an immunomodulatory and antiangiogenic agent (16) which is approved for the treatment of multiple myeloma and is increasingly used in experimental treatments of a variety of neoplastic diseases including GBM (17). It has been shown that thalidomide can down-regulate the transcription and translation of the *FGF2* gene, resulting in dramatic suppression of the anchorage-independent growth of U87 MG and other glioma cells (27).

Previously, we have demonstrated that treatment of human GBM U343 cells with RA caused increased expression of HOXB7 which was abrogated if cells were treated simultaneously with thalidomide (28). In the present study, we hypothesized that down-regulation of *FGF2* by thalidomide may enhance the radiosensitizing effect of 13-cis

RA. The purpose of the present study was to determine the effect of 13-*cis* RA and thalidomide, as well as their combination, on radiosensitivity in human GBM U343 MG cells *in vitro*.

Materials and Methods

Cell culture and drug treatment. The U343MG human glioblastoma cell line was obtained from the tumor cell bank of German Cancer Research Center, Heidelberg, Germany. Cells were grown as monolayer in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany) at 37˚C with 5% CO_2. 13-*cis* RA and thalidomide (Sigma Aldrich, Taufkirchen, Germany) were dissolved in dimethyl sulfoxide (DMSO) and aliquots were stored at –20˚C. For treatment, 1/1000th volume of 1000-fold concentrated stock solution in DMSO was added to the cell culture medium. The same volume of DMSO was added to the controls, yielding an end concentration of 0.2% DMSO.

Determination of clonogenic cell survival. The effect of drugs and IR on clonogenicity was tested using a colony-formation assay (CFA). For treatment in clonal culture, 100-5,000 cells were seeded in 25 cm^2 tissue culture flasks. Previously, we have shown that 13-*cis* RA inhibited colony formation of U343 cells in clonal culture with an half-maximal inhibitory concentration (IC_{50}) value of ~4 μM (28). Thus, we incubated U343 cells with 3 μM or 10 μM 13-*cis* RA for 1 h before irradiation. Thalidomide was added at a concentration of 100 μg/ml which causes up to 20% reduction of colony formation (28). After irradiation, cells were incubated for 17 days without medium change and with drugs present during the whole incubation time. To determine the influence of longer pre-treatment time on radiosensitivity, delayed plating experiments were performed. Briefly, 2×10^5 cells were seeded in 25-cm^2 tissue culture flasks and incubated for 24 h followed by treatment with 13-*cis* RA, thalidomide or their combination. U343 cells were more resistant to 13-*cis* RA in mass culture (28) and thus a concentration of 30 μM was used. The cells were treated in mass culture in the medium containing 10% or 1% FBS with 30 μM 13-*cis* RA 24 h before IR and 100 μg/ml thalidomide was added either simultaneously with 13-*cis* RA or delayed, 18 h later, *i.e.* 6 h before IR. After irradiation cells were incubated for 5 h to allow for recovery and then trypsinized and plated for colony formation in fresh medium containing 10% FBS without the drugs. Seventeen days later, cells were fixed with methanol/acetic acid (3:1) and stained with crystal violet dye. Colonies with more than 50 cells were scored, and plating efficiencies and surviving fractions (SF) were calculated. Cell survival curves were fitted by the linear-quadratic model, $SF=\exp[-(\alpha D+\beta D^2)]$, using SigmaPlot 8.0 Software (SPSS Inc., Chicago, IL, USA).

Quantitative polymerase chain reaction (qPCR). Cells (1×10^6) were seeded in 25 cm^2 tissue culture flasks and treated the next day with 30 μM 13-*cis* RA for 24 h and 100 μg/ml thalidomide were added either simultaneously (24 h thalidomide treatment) or delayed, 18 h after 13-*cis* RA, *i.e.* for 6 h before isolation of RNA. Isolation of total RNA was performed with the RNeasy mini Kit (Qiagen, Hilden Germany), according to the manufacturer's instructions. cDNA was generated from 1 μg total RNA using First-Strand cDNA Synthesis kit for RT-PCR (Roche Diagnostics GmbH, Mannheim, Germany) using oligo-p(dT) primer according to the manufacturer's instructions. The resulting cDNA was used for quantitative real-time PCR consisting of 10 μl 2X TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany), 8 μl RNase free water, 1 μl primers and probe and 1 μl cDNA (1:5 diluted in H_2O) per reaction. Primers and probes for *FGF2* were obtained as Assay-on-Demand gene expression products (Applied Biosystems; TaqMan FAM-MGB probe: Hs00270131_m1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Applied Biosystems; human endogeneous control, VIC-MGB probe) was used as a reference gene. All reactions were performed as singleplex and in triplicates using ABI Prism 7000 sequence detection system (Applied Biosystems) and the following temperature profile: 10 min at 9˚C, 45 cycles of 15 seconds at 9˚C and 1 min at 60˚C. Calculations of the relative transcript levels were carried out by the comparative CT method.

Irradiation. Cells were irradiated with graded doses (2-8 Gy) of 6 MV x-rays from a linear accelerator (Elekta, Hamburg, Germany) at a dose rate of 2 Gy/min.

Statistics. Mean values were compared by Student's *t*-test. The threshold of statistical significance was set at $p<0.05$.

Results

Effects of 13-cis RA and thalidomide on radiosensitivity in clonal culture. The influence of 13-*cis* RA and thalidomide on the radiosensitivity of U343 cells was first tested for irradiation in clonal culture. The radiosensitivity did not change significantly under these conditions (Figure 1A) compared to that of untreated cells. In Figure 1B we show that treatment of cells with thalidomide 1 h before IR resulted in a trend towards radioprotection.

Effects of 13-cis RA and thalidomide on FGF2 gene expression. Because the expected enhancement of the radiosensitivity by 13-*cis* RA and thalidomide was not observed, we tested the influence of these drugs on *FGF2* gene expression. *FGF2* expression was up-regulated after 6-12 h treatment with 13-*cis* RA and remained elevated at 12-24 h (Figure 2A). Treatment with 100 μg/ml thalidomide for 6 h did not significantly change *FGF2* expression relative to that of the untreated cells, while treatment for 24 h caused a slight increase of *FGF2* expression. Delayed addition of thalidomide was associated with a significant decrease ($p=0.007$) of 13-*cis* RA-induced *FGF2* expression (Figure 2B).

Treatment with 13-cis RA and thalidomide in mass culture. Since up-regulation of *FGF2* expression was only observed 12-24 h after the addition of 13-*cis* RA, we tested if longer pre-treatment time with the drug might lead to more effective enhancement of radiosensitivity. Based on the effect of thalidomide on 13-*cis* RA-induced *FGF2* expression described above, delayed treatment was used, *i.e.* thalidomide was added 18 h after 13-*cis* RA (6 h before IR). 24 h treatment with 30 μM 13-*cis* RA resulted in a 31±8% (mean±SE) reduction in plating efficiency without IR but did not alter radiosensitivity (Figure 3A). By contrast, treatment

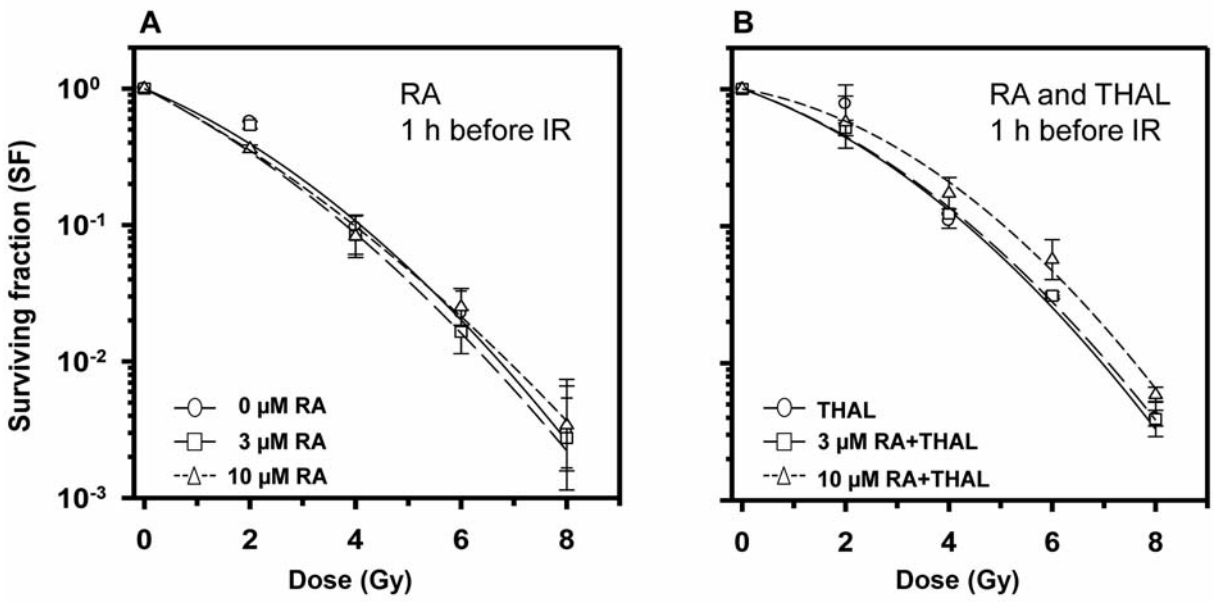

Figure 1. *Cell survival curves of U343 cells after treatment with 13-cis RA (RA) and thalidomide (THAL). A: Cells were treated in clonal culture conditions with 3 or 10 µM RA 1 h before irradiation (ionizing radiation, IR). B: 100 µg/ml THAL was added alone or in combination with RA 24 h before irradiation (simultaneous addition). Data are mean values of two independent experiments with error bars representing the half range.*

with thalidomide was slightly radioprotective (Figure 3B). However, this effect was eliminated by combined treatment with 30 µM 13-*cis* RA (Figure 3 B), although the radiosensitivity was not changed compared with the control or treatment with 13-*cis* RA alone.

These results suggest that some interaction between 13-*cis* RA and thalidomide might indeed exist. We speculated that growth factors in the culture medium might mask a down-regulation of autocrine growth factor production by thalidomide. Therefore, we tested the effect of reducing the FBS concentration in the medium from 10% to 1% during treatment. RA significantly enhanced the radiosensitivity of U343 when cells were treated in 1% FBS (p=0.04) but not in 10% FBS, supporting the idea that factors present in the serum may mask or prevent the radiosensitizing effect of 13-*cis* RA. However, addition of thalidomide did not enhance radiosensitivity further, suggesting that autocrine expression of *FGF2* induced by 13-*cis* RA was not protective under these conditions. On the other hand, thalidomide was not radioprotective in combination with 13-*cis* RA when added 6 h before IR.

Discussion

In the present study, we tested if combined treatment with 13-*cis* RA and thalidomide may enhance the radiosensitivity of U343 cells to a greater extent than treatment with these as single compounds. This was based on the hypothesis that radiosensitization by retinoids may be limited by protective mechanisms such as induction of *FGF2*. Thalidomide has been shown to down-regulate certain growth factors and cytokines, including *FGF2* (22, 27) and *interleukin-8* (*IL-8*) (44) which may contribute to development of radioresistance (1, 19, 42). Therefore, combined treatment with thalidomide might be expected to enhance radiosensitization by 13-*cis* RA.

No radiosensitization by 13-*cis* RA was found for 1 h pre-treatment with 3-10 µM 13-*cis* RA in clonal culture or for 24 h pre-treatment with 30 µM in mass culture. Thalidomide given alone did not enhance the radiosensitivity and was even slightly radioprotective under both conditions. Combined treatment with 13-*cis* RA and thalidomide did not enhance the radiosensitivity beyond that of cells irradiated in the presence of DMSO without the drugs. Up-regulation of *FGF2* expression was confirmed 12-24 h after the addition of 13-*cis* RA and this was prevented by the addition of thalidomide 18 h later, *i.e.* 6 h prior to irradiation. However, although the rationale concerning the effects of 13-*cis* RA and thalidomide on *FGF2* gene expression was indeed validated, the anticipated synergistic effect of the two drugs on radiosensitivity was not observed under standard culture conditions.

On the other hand, a significant enhancement of radiosensitivity by 13-*cis* RA alone was found when U343 cells were treated for 24 h in serum-reduced medium (1% *versus* 10% FBS). Thus, protective factors in the serum appeared to mask the radiosensitizing effect of RA but induction of autocrine *FGF2* expression did not seem to be involved. In U373 glioma cells, increased sensitivity toward ionising irradiation was observed if cells were treated with 13-

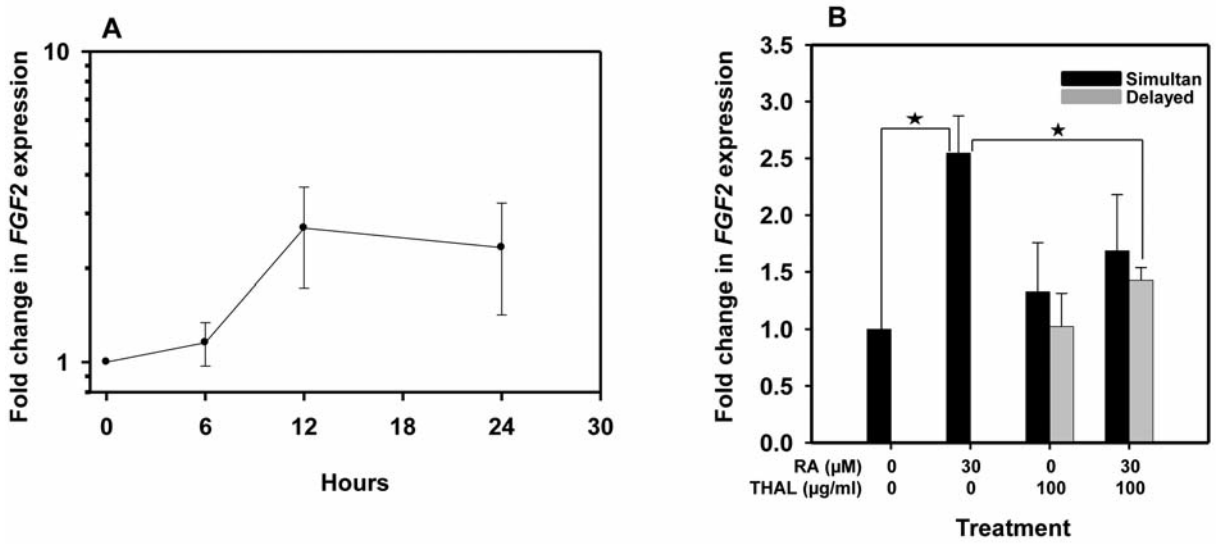

Figure 2. *Fibroblast growth factor-2 (FGF2) gene expression measured by a quantitative polymerase chain reaction (qPCR) before and after treatment of U343 cells with 13-cis RA (RA) and thalidomide (THAL). A: Kinetics of transcription of FGF2 after treatment with 30 μM RA (6, 12, and 24 h). Data are mean values from two independent experiments with error bars representing the half range. B: Fold change in FGF2 expression after treatment of U343 cells with 30 μM RA or 100 μg THAL. The cells were incubated with RA for 24 h. THAL was added at the same time as RA (simultaneously) or 18 h after treatment with RA (delayed). Zero values of concentrations imply that DMSO was added without the drug. For 0 μg/ml THAL, only simultaneous addition of DMSO was included (hence only one column). Data are mean values and standard error of the mean from three independent experiments. An asterisk (*) signifies p<0.05.*

cis RA in combination with interferon while treatment with 13-*cis* RA as a sole agent did not have clear effect on the radiosensitivity of the same cells (26). However, the precise mechanism responsible for these findings was not investigated.

Moderate radiosensitization by retinoids has been found in different cell types (21, 43). In human squamous cell carcinoma, the radiosensitizing effect of retinoids correlated with cellular retinoic acid-binding protein-1 (CRABP1) protein expression level (21). Cells with high basal expression of CRABP1 were more radioresistant to combined treatment with 13-*cis* RA and IR than cells with low basal expression. Overexpression of this protein in 13-*cis* RA-sensitive cells caused a more resistant phenotype, independent of RAR-2 expression. CRABP1 overexpression induced stimulation of expression of cyclin D1, an important G-1/S-specific cell- cycle protein (6). It has been reported that RA also increased G_1 delay in radiosensitive SiHa cervical cancer cells, indicating that the radiosensitizing effect of 13-*cis* RA may be a consequence of its modulation of the cell cycle distribution (5). In cervical cancer cell lines, treatment with 13-*cis* RA caused *c-Fos* mRNA repression which may be related to the observed increase of radiosensitivity (43). Interestingly, the same authors found that the mechanism of 13-*cis* RA radiosensitization did not require for functional p53 protein.

In GBM, other possible mechanisms which may contribute to radioresistance are EGFR overexpression (3) or a glioma-specific *EGFR* mutation EGFRvIII (29). Based on evidence that 13-*cis* RA may inhibit *EGFR* activity in glioma cells (39, 46) we had anticipated a sensitizing effect of 13-*cis* RA. It has been shown that treatment of GBM cells with EGFR-specific inhibitory agents such a gefitinib or cetuximab, led to increased sensitivity toward IR (14, 38). Several *in vitro* studies suggest that IR mimics the action of growth factor ligand-receptor binding by activating downstream signaling (18, 33). It has been demonstrated that after IR, EGFR can translocate into the nucleus where it may act as a transcription factor (13, 23) and increase the activity of DNA-dependent protein kinase, which plays an important role in DNA repair processes (13).

In our previous work, we demonstrated that treatment of human GBM U343 cells with 13-*cis* RA caused increased expression of *HOXB7* which was abrogated if cells were treated simultaneously with thalidomide (28). In the present experiments, we observed no significant effect of thalidomide on *FGF2* expression qPCR if cells were treated with thalidomide for 6 h, while treatment for 24 h caused an increase in *FGF2* expression. Furthermore, the lack of effect of thalidomide on *FGF2* expression in the case of simultaneous treatment with both drugs may be explained by the short half-life of thalidomide, which is approximately 2 h under *in vitro* conditions (31). Thus, at the time when *FGF2* expression was up-regulated (6-12 h after treatment with 13-*cis* RA), most thalidomide would be degraded. In delayed treatment with thalidomide (18 h after treatment with 13-*cis* RA), when *FGF2* was already up-regulated, down-regulation of *FGF2* by thalidomide was observed. In our experiments,

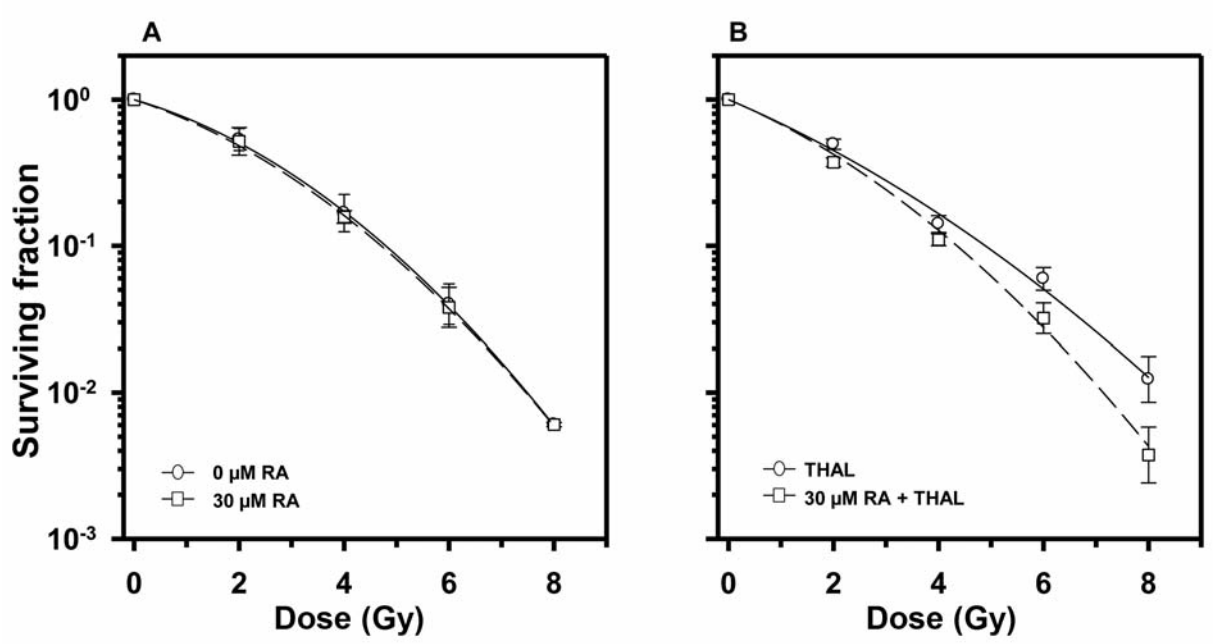

Figure 3. *Cell survival curves of U343 cells after combined treatment with 13-cis RA (RA; 30 µM) 24 h and thalidomide (THAL; 100 µg/ml) 6 h before irradiation with ionizing radiation (IR) under mass culture condition. After harvesting and seeding, cells were incubated for colony formation in medium without drugs. A: The effect on radiosensitivity of U343 cells of 24 h treatment with RA-alone. Data are mean values ± standard error of the mean from five independent experiments. B: The effect of combined treatment with RA (24 h) and THAL (6 h) on radiosensitivity of U343 cells. Data are mean values ± standard error of the mean from three independent experiments. Zero concentration, or no drug, implies that DMSO was added without the drug.*

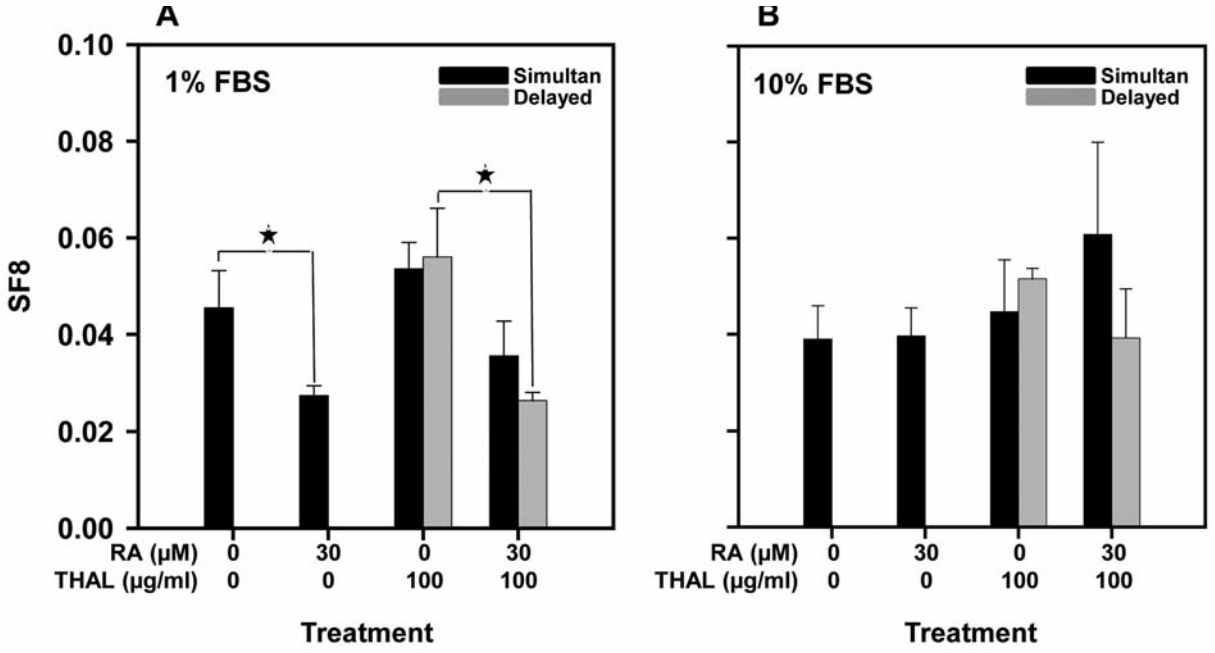

Figure 4. *The effect of medium containing 1% (A) or 10% (B) fetal bovine serum (FBS) 13-cis RA (RA) with and without thalidomide (THAL) on radiosensitivity of U343 cells irradiated with 8 Gy. Cells were treated in mass cultures with 30 µM RA 24 h before IR with 8 Gy. THAL was added at the same time as RA 24 h before IR (simultaneously) or 18 h later (delayed), i.e. 6 h before IR. Zero values of concentrations mean that DMSO was added without the drug. For 0 µg/ml THAL, only simultaneous addition of DMSO was included (hence only one column). Data are mean values ± standard error of the mean from three independent experiments. An asterisk (*) signifies $p<0.05$. (SF8, Survival fraction after irradiation with 8 Gy).*

we found that treatment of cells with thalidomide tended to being radioprotective. Thalidomide has diverse effects on a number of targets (4) which might also contribute to protecting of the cells. For example, thalidomide reduces the activity of the inflammatory cytokine tumor necrosis factor-α by accelerating the degradation of its mRNA (25). This cytokine may be induced by IR in tumor cells, where it plays an important role in radiation-induced apoptosis (12).

The efficacy of 13-*cis* RA and thalidomide as sole agents was investigated in clinical studies in patients with newly-diagnosed or recurrent GBM (15, 35) and as single-agents in combination with other chemotherapeuticals (11) or RT (2, 7). The results did not demonstrate any benefit compared to standard therapy. A combination of 13-*cis* RA and thalidomide has only been investigated clinically in a single phase I factorial design study on adjuvant therapy after radiochemotherapy with RT and temozolomide for newly-diagnosed GBM (17).

We conclude that 13-*cis* RA has the potential to enhance the radiosensitivity of U343 cells. However, the effect was seen only when FBS was reduced to 1% during and after irradiation and was masked when using 10% FBS, suggesting the presence of protective factors in FBS. While thalidomide was able to abrogate 13-*cis* RA-induced expression of *FGF2*, it did not enhance radiosensitivity when combined with 13-*cis* RA. Although the two drugs did not have the anticipated synergetic effect on radiosensitivity, we previously found an additive effect on proliferation (28). Since a radioprotective effect of thalidomide was not observed when combined with 13-*cis* RA, it should be possible to exploit the anti-proliferative effect of the combination without compromising the radiosensitivity of the tumor cells. Thus, further studies on the interaction of these drugs with radiochemotherapy are warranted.

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