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Plasminogen Kringle 5 Induces Apoptosis of Brain Microvessel Endothelial Cells: Sensitization by Radiation and Requirement for GRP78 and LRP1

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Abstract

Recombinant plasminogen kringle 5 (rK5) has been shown to induce apoptosis of dermal microvessel endothelial cells (MvEC) in a manner that requires glucose-regulated protein 78 (GRP78). As we are interested in anti-angiogenic therapy for glioblastoma tumors, and the effectiveness of anti-angiogenic therapy can be enhanced when combined with radiation, we investigated the pro-apoptotic effects of rK5 combined with radiation on brain MvEC. We found that rK5 treatment of brain MvEC induced apoptosis in a dose- and time-dependent manner, and that prior irradiation significantly sensitized (500-fold) the cells to rK5-induced apoptosis. The rK5-induced apoptosis of both unirradiated and irradiated MvEC required expression of GRP78 and the low density lipoprotein receptor-related protein 1 (LRP1), a scavenger receptor, based on downregulation studies with small interfering RNA, and blocking studies with either a GRP78 antibody or a competitive inhibitor of ligand binding to LRP1. Furthermore, p38 MAP kinase was found to be a necessary downstream effector for rK5-induced apoptosis. These data suggest that irradiation sensitizes brain MvEC to the rK5-induced apoptosis and that this signal requires LRP1 internalization of GRP78 and the activation of p38 MAP kinase. Our findings suggest that prior irradiation would have a dose-sparing effect on rK5 anti-angiogenic therapy for brain tumors and further suggest that the effects of rK5 would be tumor-specific as the expression of GRP78 protein is upregulated on the brain MvEC in glioblastoma tumor biopsies as compared to the normal brain.

Keywords

Brain endothelial cells; plasminogen; Kringle 5; LRP1; GRP78; apoptosis

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INTRODUCTION

Glioblastoma tumors have a dismal prognosis with a median survival of 12 to 15 months. As the tumors typically exhibit angiogenesis (1–3), anti-angiogenic therapy may represent an effective therapeutic strategy. In this study we investigated the pro-apoptotic effect of the recombinant form of the fifth kringle domain of plasminogen (rK5) on human brain microvessel endothelial cells (MvEC). Irradiation is known to promote the ability of other anti-angiogenic agents to inhibit tumor growth (4–6) and irradiation is a standard initial therapy for patients with glioblastoma tumors (2); therefore, we also investigated the potential promotion of rK5-induced apoptosis of brain MvEC by irradiation.

It has been shown previously that rK5 induces changes indicative of apoptosis in non-brain MvEC (7,8) and inhibits proliferation of basic fibroblast growth factor (bFGF)-stimulated calf pulmonary arterial endothelial cells and bovine adrenal capillary endothelial cells (8,9). The ability of rK5 to inhibit neovascularization has been demonstrated directly in a rat model of hyperoxia-induced retinal neovascularization (10). Moreover, stable expression of K5 in U-87MG glioblastoma cells prior to their s.c. propagation in nude mice resulted in inhibition of angiogenesis and tumor growth (11) and inhibition of angiogenesis was seen when colorectal carcinoma cells stably expressing K5 were propagated s.c. in athymic nude mice (12).

The cell surface binding protein for rK5 on dermal MvEC is glucose-regulated protein 78 (GRP78) (7). GRP78 is a member of the heat shock protein (HSP) 70 family, and its upregulation is part of the general cellular defense mechanism of stressed cells that is referred to as the unfolded protein response (reviewed in 13); (14). The increased expression of members of the unfolded protein response in tumors suggests they may be promising therapeutic targets (reviewed in 15). GRP78 associates with the scavenger receptor low density lipoprotein receptor-related protein 1 (LRP1) on the cell surface (16). LRP1 is known to signal upon associating with and internalizing its ligands (17–20). The biologic consequences of its internalization are varied and depend on which one of approximately 30 different ligands it binds (reviewed in 21,22); (23), as well as its association with other cell surface proteins, the cell type, and the experimental conditions (16,20,24,25). As the rK5 inhibition of retinal capillary endothelial cell proliferation was unaffected by RGD-containing peptides (10), it likely occurs in an integrin receptor-independent manner.

We found that rK5 induces apoptosis of brain MvEC and that prior irradiation significantly sensitizes the cells to rK5-induced apoptosis resulting in a dose-sparing effect. In both unirradiated and irradiated brain MvEC, the rK5-induced apoptosis requires expression of GRP78 and LRP1, as well as the activation of p38 mitogen-activated protein (MAP) kinase.

MATERIAL AND METHODS

Materials

Recombinant K5 (rK5) (ABT-828) expressed in yeast was provided by Abbott Laboratories (Abbott Park, IL); its native folded structure was verified by NMR as being comparable to K5 naturally derived from human plasminogen by elastase cleavage. PD98059 was purchased from Calbiochem, and SB202190 and SP600125 from A.G. Scientific Inc.. Recombinant receptor-associated protein (RAP) was purchased from Maine Biotechnology Services Inc. (Portland, ME), and dialyzed prior to use.

Cell culture

Primary human brain MvEC were purchased from Cell Systems, utilized at passages 2 to 8 and propagated as recommended in 70% CSC media and 30% M199 media, with 10% low-

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endotoxin FBS (BD Biosciences) Unless otherwise indicated, cells were split the day before the experiment, and then re-plated onto collagen-coated wells in M199 media with 10\% low endotoxin FBS, 5 ng/ml bFGF and 10 ng/ml vascular endothelial growth factor (VEGF) 4 h prior to treatment with rK5. Cells were subconfluent at the time of treatment.

**Analysis of cell surface expression**

For analysis of the cell surface expression of GRP78 and LRP-1, cells were labeled with biotin (26), lysed in 1\% NP40 lysis buffer (20 mM TrisBase, 137 mM NaCl, 2 mM EDTA, pH 8.0 with 10\% glycerol and with protease inhibitors, immunoprecipitated with mAb anti-LRP1 or goat anti-GRP78 IgG, the immunoprecipitates harvested by centrifugation, washed, subjected to SDS-PAGE, transferred to an Immobilon-P membrane, reacted with HRP-conjugated streptavidin, and developed using chemiluminescence (Amersham).

Alternatively, cells were incubated with 10 \( \mu \text{g/ml} \) of primary antibody (30 min, 4\°C), washed, incubated with 10 \( \mu \text{g/ml} \) Alexa-488-conjugated secondary antibody (30 min, 4\°C), washed, fixed, and subjected to FACS analysis using a FACScan instrument, as described previously (27). Goat anti-GRP78 (directed toward the N-and C-terminus) was purchased from Santa Cruz Biotechnology and mAb anti-LRP1 from Calbiochem.

**Immunoblot analysis**

Cells and tissue samples were lysed in RIPA lysis buffer (0.05 M Hepes, pH 7.4, 0.15M NaCl, 1\% deoxycholate, 1\% Triton X-100 and 0.1\% SDS) with protease inhibitors, as described previously (28). Equivalent amounts of lysate (typically 100–130 \( \mu \text{g} \)) were separated on SDS-PAGE then transferred to an Immobilon-P membrane, probed with primary antibody (4\°C, overnight), washed, reacted with a horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad), and developed using enhanced chemiluminescence (Amersham) (28). Antibodies were purchased as follows: rabbit anti-p38 MAP kinase, anti-phospho-p38 MAP kinase (Y182), anti-HSP70, anti-c-terminal Jun kinase (JNK), and anti-calreticulin (Santa Cruz Biotechnology); rabbit anti-cleaved caspase-3 or 7 (Calbiochem); rabbit anti-von Willebrand factor (vWF IgG) (Chemicon); mAb anti-actin (Sigma), mouse anti-caspase-3 (Cell Signaling); and mAb anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Research Diagnostics Inc.).

**siRNA downregulation**

SMARTpool small interfering (si)RNA consisting of a pool of four SMARTselection-designed siRNA duplexes directed toward GRP78, HSP70, calreticulin, p38 MAPK, and extracellular signal-regulated kinase 1 (Erk1) were purchased from Dharmacon, and siRNA directed towards LRP1 was purchased from Santa Cruz Biotechnology. HP-validated siRNA directed toward JNK2 was purchased from Qiagen. Two nonsense mutations were introduced into the JNK2 siRNA sequence as a custom control purchased from Qiagen. Transient transfections were carried out using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s guidelines. The addition of liposome without siRNA was administered in control conditions.

**Apoptosis assays**

Terminal 3’-dexoynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were carried out using the ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon) as per the manufacturer’s instructions and as described previously (28). Tumor necrosis factor \( \alpha \) (TNF\( \alpha \)) induction of apoptosis was used as a positive control (29). For determination of caspase 3/7 activity, a caspase-3/7 luminescent activity assay was performed using the Caspase-Glo 3/7 Assay kit (Promega).
Analysis of human tissue samples
Tissue samples were obtained from the Cooperative Human Tissue Network of the National Cancer Institute and the University of Alabama at Birmingham Brain Tumor Bank in accordance with University Human Tissue Committee policies. Tumors were histologically graded according to the World Health Organization classification of brain tumors (1). Frozen normal adult brain (cortex and white matter) and glioblastoma tumor samples obtained at autopsy within 18 h of death were homogenized in RIPA lysis buffer with protease inhibitors as described (30) for western blot analysis. For immunohistochemical analysis, frozen sections, as well as formalin- fixed and paraffin-embedded, normal brain and glioblastoma tumor samples were prepared from surgical biopsy samples and treated with blocking buffer to inhibit endogenous peroxidases and prevent non-specific protein binding, reacted with the primary antibody in 5% BSA/PBS/0.01% Tween 20 (4°C, 20 h), washed, reacted with a HRP-conjugated secondary antibody (22°C, 1 h), followed by the 3,3’-diaminobenzidine (DAB) substrate (ScyTek) (30), then counterstained with hematoxylin.

Statistics
After determining the data were normally distributed a two-sample t test was used for data analysis and a p < 0.05 was considered significant.

RESULTS
rK5 induces apoptosis of primary human brain MvEC
rK5 treatment has been shown to induce apoptosis of dermal MvEC (7). To determine the potential of rK5 treatment to induce apoptosis of human brain MvEC, we treated primary human brain MvEC plated onto collagen type IV and grown in complete media (10% FBS) in the presence of VEGF (10 ng/ml) and bFGF (5 ng/ml) with rK5 at concentrations of 10 to 5000 ng/ml for 17 h. We found that 5000 ng/ml of rK5 was necessary to induce a significant increase in the numbers of TUNEL-positive human brain MvEC (≈ 8% positive) at 17 h (Fig.1A). Immunoblotting analysis as well as caspase 3/7 activity analysis confirmed that treatment of the cells with 5000 ng/ml rK5 for 17 h resulted in a significant increase in cleaved caspase-7, and that treatment for time periods greater than 17 h (25 and 40 h) did not result in a further increase in the amounts of cleaved caspase-7 (data not shown).

Irradiation sensitizes primary human brain MvEC to rK5-induced apoptosis
To evaluate the effect of prior irradiation on rK5-induced apoptosis of brain MvEC, we irradiated (2 or 5 Gy) primary human brain MvEC, allowed the cells to recover for 20 h, then re-plated them onto collagen-coated wells for 4 h prior to treatment with rK5 for 17 h. In the absence of treatment with rK5, only a low percentage of brain MvEC that were irradiated exhibited TUNEL positivity (Fig. 1B). Notably, the percentage of TUNEL-positive cells in the brain MvEC that had been irradiated prior to treatment with 10 ng/ml of rK5 was similar to the percentage of TUNEL-positive cells in unirradiated MvEC that were treated with 5000 ng/ml of rK5 (Fig. 1B). Thus, the prior irradiation appeared to increase the sensitivity of the cells to rK5, i.e., the irradiation had a dose sparing effect, but did not seem to increase the numbers of cells that were susceptible to rK5. Similarly, irradiation of the brain MvEC (2 or 5 Gy) followed by rK5 treatment (3 – 5000 ng/ml, 17 h) resulted in a significant increase in the cleavage of caspase-3 or 7 that was maximal at 10 ng/ml rK5 (Fig. 1C, and data not shown) confirming that irradiation plus rK5 induces apoptosis. Consistent with the results of the TUNEL assays, irradiation alone (5 Gy) did not induce a significant increase in cleaved caspase-3 (Supplemental Fig. 1A). The time course of rK5-induced caspase-7 cleavage in the irradiated brain MvEC was maximal at 17 h and detectable as...
early as 3 h post-rK5 treatment (Fig 1D). Collectively, these data establish that prior irradiation of the brain MvEC resulted in a significant reduction in the dose of rK5 required for optimal induction of apoptosis.

**GRP78 is necessary for rK5-induced apoptosis of unirradiated and irradiated primary human brain MvEC**

Analysis of the expression of GRP78 on the cell surface of the MvEC used in the analyses of rK5-induced apoptosis was determined by cell surface biotinylation followed by detergent lysis, immunoprecipitation with anti-GRP78 antibody and SDS-PAGE analysis. The data indicated that GRP78 was expressed on both unirradiated and irradiated human brain MvEC (Fig. 2A). Although irradiation has been reported to upregulate GRP78 (31), we found that the surface expression was equivalent in the irradiated and unirradiated brain MvEC (Fig 2A) suggesting that an upregulation of GRP78 does not contribute to the enhanced sensitivity of the irradiated brain MvEC to low doses of rK5 in our experiments.

After further confirming the cell surface expression of GRP78 on the brain MvEC by FACS analysis (data not shown), we determined the requirement for GRP78 in rK5-induced apoptosis by downregulating GRP78 with specific duplex siRNA. As a control, we downregulated the endoplasmic reticulum chaperone protein calreticulin, which is expressed, in part, on the cell surface (19) or we downregulated HSP70. Downregulation of GRP78 and calreticulin was confirmed by western blotting (Supplemental Fig. 1B) and we had established previously that HSP70 protein is downregulated by >70% with 50 nM siHSP70 treatment in the unirradiated human brain MvEC (data not shown). No adverse effects of the siRNAs on cell viability or morphology were detected over the time course of these experiments (data not shown). The downregulation of GRP78 significantly blocked rK5-induced apoptosis (5000 ng/ml; 17 h) of the unirradiated brain MvEC as determined using a TUNEL assay and blotting for cleaved caspase-3 (Fig. 2B), whereas the downregulation of calreticulin or HSP70 had no effect.

Similar results were obtained on downregulation of GRP78 in irradiated cells. Downregulation of GRP78 (> 70%) was achieved on treatment of the irradiated brain MvEC with 50 nM siGRP78 (Supplemental Fig. 1C). Downregulation of GRP78 in the irradiated brain MvEC significantly blocked rK5-induced apoptosis (10 ng/ml rK5; 17 h) as detected using the TUNEL assay and blotting for cleaved caspase-7 (Fig 2C). The downregulation of HSP70 had no effect. Further support for a role for GRP78 in rK5-induced apoptosis of the irradiated brain MvEC was obtained by the results of treatment with an anti-GRP78 antibody (10 µg/ml) that is directed toward the N-terminus. This inhibited rK5-induced apoptosis (data not shown) as described previously for unirradiated dermal MvEC (7). These data indicate that GRP78 is necessary for rK5-induced apoptosis of both unirradiated and irradiated primary human brain MvEC.

**LRP1 is necessary for rK5-induced apoptosis of unirradiated and irradiated human brain MvEC**

GRP78 is known to associate with LRP1 on the cell surface (16); therefore we examined the potential role of LRP1 in rK5-induced apoptosis of MvEC. The expression of LRP1 on the cell surface was verified using cell surface biotinylation, followed by detergent lysis and immunoprecipitation with a mAb anti-LRP1, which recognizes the 85-kDa light chain. SDS-PAGE analysis of the immunoprecipitates indicated that LRP1 is expressed on the surface of both the unirradiated and irradiated MvEC, and further indicated the levels of expression of LRP1 on the cell surface were not altered by irradiation (Supplemental Fig. 2A). To determine whether LRP1 is required for the rK5-induced apoptosis of the human brain MvEC, we pretreated the cells with receptor associated protein (RAP), a competitive

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inhibitor of ligand binding to LRP1 (32). Prior addition of 60 nM rec-RAP blocked rK5-induced apoptosis in the irradiated and unirradiated cells as detected using the TUNEL assay and cleavage of caspase-3 (Fig 3A&B, respectively). These results were confirmed by downregulation of LRP1 with pooled specific duplex siRNA. Downregulation was confirmed by western blotting of the irradiated and unirradiated cells (Supplemental Fig. 2B&C, respectively) and no effect of siLRP1 administration on cell viability or morphology was detected over the time course of these experiments. Downregulation of LRP1 post-irradiation blocked rK5-induced apoptosis (10 ng/ml), whereas the downregulation of HSP70 as a control had no effect (Fig 3C). In the unirradiated cells, downregulation of LRP1 significantly blocked the rK5-induced apoptosis (5000 ng/ml), whereas the downregulation of calreticulin had no effect (Fig 3D). These data suggest that LRP1 internalization of GRP78 is likely necessary for rK5-induced apoptosis in both the irradiated and the unirradiated brain MvEC.

**p38 MAP kinase is necessary for rK5-induced apoptosis of the irradiated human brain**

**MvEC**

Stress is known to induce the activation of p38 MAP kinase (33) and p38 MAP kinase can promote a pro-apoptotic signal (34–36). We found a time-dependent increase in phosphorylated p38 MAP kinase in the irradiated cells treated with rK5 (Fig. 4A). Treatment with a small molecule inhibitor of p38 MAP kinase (SB202190) at 2.5-fold the IC50 (1.5 µM), significantly blocked the pro-apoptotic effect of rK5 in the irradiated brain MvEC (Fig. 4B). In contrast, the pro-apoptotic effects of rK5 were not blocked by treatment with inhibitors of other kinases at 2.5-fold the IC50: 5 µM PD98059 (MEK inhibitor), SP600125 (JNK inhibitor), and FR180204 (Erk inhibitor) (Fig 4B). To further evaluate the requirement for p38 MAPK in the irradiated MvEC we downregulated p38 MAPK with pooled specific duplex siRNA and downregulated Erk1 as a control (Supplemental Fig. 3A). TUNEL analysis indicated that the downregulation of p38 MAPK in the irradiated brain MvEC blocked the pro-apoptotic effect of rK5 (Fig. 4C), whereas the downregulation of Erk1 had no effect.

On analysis of the unirradiated cells, we also found that the p38 MAPK inhibitor SB202190 at 2.5 fold the IC50 significantly blocked the pro-apoptotic effect of rK5 (Fig 5A, lanes 2&3) whereas the Erk inhibitor FR180204 did not (Fig 5A, lanes 2&4). Similarly, downregulation of p38 MAP kinase in the unirradiated cells (Supplemental Fig. 3B) blocked the pro-apoptotic effect of rK5 as detected by TUNEL assay, whereas the downregulation of Erk1 had no significant effect (Fig 5B). These data suggest that p38 MAP kinase is a necessary downstream signaling effector in the pro-apoptotic effect of rK5 in irradiated and unirradiated human brain MvEC.

Although JNK has been reported to be involved in signaling that promotes apoptosis (34), we found that the JNK inhibitor, SP600125, at 2.5-fold the IC50 in the irradiated cells (Fig. 4B) or the unirradiated cells (Fig. 5A) did not block the pro-apoptotic effect of rK5. However, a small increase (30%) in phosphorylation of the 54-kDa JNK2 isoform was detected in a time course analysis of JNK phosphorylation post-rK5 treatment of the unirradiated cells (data not shown). Downregulation of JNK2 with 200 nM specific duplex siJNK2 RNA (70% downregulation of the 54-kDa isoform) followed by TUNEL assay confirmed that downregulation of JNK2 in the unirradiated cells did not block the pro-apoptotic effect of rK5 (5000 ng/ml) (data not shown).
**GRP78 expression is higher on brain MvEC in glioblastoma tumor samples as compared to the normal brain**

As our ultimate goal is to develop a novel therapeutic strategy for glioblastoma tumors, we evaluated the expression of GRP78 in frozen and paraffin sections of 14 glioblastoma tumor biopsy samples by immunohistochemistry. We found moderate (2+) expression of GRP78 in an estimated 30% of MvEC in the tumor portion of all glioblastoma samples (n=14) and moderate (2+) expression in 30% of tumor cells (Fig. 6A). The staining localized to the cell membrane and the cytoplasm. The staining was specific for GRP78 as it was largely competed out by preincubation of the anti-GRP78 antibody with a 50-fold molar excess of GRP78 peptide (data not shown). Staining with rabbit IgG was used as a negative control and staining with anti-vWf antibody was used as a positive control. Using this technique GRP78 expression was below the limit of detection in endothelial and glial cells in the frozen and paraffin sections of normal brain (n=13), although low (1+) expression of GRP78 was detected in neurons in the normal brain (data not shown). To confirm the upregulation of GRP78 in the tumors as compared to the normal brain, we immunoblotted lysates from nine normal brains and nine glioblastoma tumor samples with anti-GRP78 antibody, followed by stripping and reprobing with antibodies directed toward GAPDH and HSP70. The immunoblotting data were quantitated by densitometric analysis and GRP78 expression was normalized to HSP70 and to GAPDH and plotted as bar graphs (Figure 6B). We found on average a 2.0 to 2.5-fold increase in GRP78 expression relative to HSP70 or when normalized to GAPDH in the nine tumor samples as compared to the nine normal brain (NB) samples (GRP78/HSP70 densitometry ratio: NB mean = 0.38 ± 0.06, GBM mean = 0.92 ± 0.2 with p<0.05; GRP78/GAPDH densitometry ratio: NB mean = 0.36 ± 0.05, GBM mean = 1.15 ± 0.3 with p<0.05). These data support our immunohistochemistry results indicating that GRP78 expression is upregulated in glioblastoma tumors in vivo.

**DISCUSSION**

In this report we show that rK5 can induce apoptosis of brain MvEC and that irradiation significantly sensitizes primary human brain MvEC to the pro-apoptotic effect of rK5. We found that rK5 treatment of brain MvEC induces apoptosis, as measured by several different assays, when administered at 5000 ng/ml (≈ 500 nM). This is similar to the dosage of rK5 required to induce apoptosis of dermal MvEC (7). Importantly, we found that prior irradiation significantly sensitized the brain MvEC to rK5-induced apoptosis, as a 500-fold lower dose of rK5 (10 ng/ml) induced a similar percentage of TUNEL-positive cells and cleavage of caspase-3 or 7.

Few studies have focused on irradiation as a potential sensitizing agent for anti-angiogenic therapy. Our studies are consistent with the recent report of Jin et al., (37) that rK5 combined with irradiation enhanced the anti-angiogenic effect of rK5 in a mouse tumor model of Lewis lung carcinoma cells propagated s.c.. Under our experimental conditions (10% FBS with 10 ng/ml VEGF and 5 ng/ml bFGF) irradiation alone did not induce significant cell death or caspase 3/7 cleavage. Other investigators have reported a pro-apoptotic effect of irradiation (2 or 5 Gy) alone on dermal MvEC that were propagated and radiated in reduced serum (2% FBS), and this was attributed to reduced levels of the anti-apoptotic protein Bcl2 (38,39). We did not find an altered level of Bcl2 protein post-irradiation of the MvEC at two time points in our experimental conditions (B.C. McFarland and C.L. Gladson, unpublished observation). This may be due to the higher levels of FBS and added VEGF, as the level of Bcl2 expression is known to be regulated by VEGF (39) or it may reflect the use of brain MvEC rather than dermal MvEC. rK5 has been reported to induce autophagy of non-brain endothelial cells in different experimental conditions (40); therefore, it is possible that rK5-induced autophagy contributes to the apoptosis we have observed in the brain MvEC.
Our finding that GRP78 is required for rK5-induced apoptosis of human brain MvEC is consistent with the report of the requirement for GRP78 in the pro-apoptotic effect of rK5 on dermal MvEC, and of a direct interaction of rK5 with rec-GRP78 (7). GRP78 is an endoplasmic reticulum chaperone protein that is expressed on the cell surface in stress conditions and in tumors (14,15); however, we found no change in the cell surface expression post-irradiation in the time course and conditions of our experiments. This suggests that an increase in GRP78 expression post-irradiation is not the mechanism by which irradiation sensitizes the cells to rK5-induced apoptosis. The ligand binding to cell surface GRP78 appears to determine the signal generated. We and others (7) detect a pro-apoptotic signal when rK5 binds to cell surface GRP78 on MvEC, whereas α2-macroglobulin binding to cell surface GRP78 on human prostate cancer cells and macrophages initiates a pro-proliferation signal (41). The voltage-dependent ion channel also has been reported to be a receptor for rK5 (42).

We found that LRP1 is necessary for the rK5-generated pro-apoptotic signal. This suggests that LRP1 internalization of GRP78 is likely necessary for the pro-apoptotic effect of rK5 in the brain MvEC. The signal generated upon LRP1 binding and internalizing its ligand is dependent on the cell and the environmental context (21). For example, a pro-migratory signal is generated when LRP1 binds and internalizes the complex of cell surface calreticulin and thrombospondin-1 in coronary artery endothelial cells (19,20). Differential LRP1 signaling is thought to be due to different adaptor molecules that bind to the phosphorylated tyrosine residue(s) in the LRP1 cytoplasmic tail and thereby activate different downstream effectors (21,23,43).

We found that p38 MAP kinase was activated with rK5 treatment post-irradiation and that p38 MAP kinase was necessary for rK5-induced apoptosis of the irradiated and unirradiated brain MvEC. In contrast, neither Erk nor JNK were necessary for the rK5-induced apoptosis of the human brain MvEC. Notably, the LRP1 family member LRP8, expressed on platelets, is known to activate p38 MAP kinase upon binding its ligand, the low density lipoprotein, suggesting that the LRP family of proteins is capable of signaling to p38 MAP kinase (44).

In support of the potential clinical relevance of our findings, we show that the expression of GRP78 is increased on the MvEC in glioblastoma tumor samples as compared to the normal brain. GRP78 expression was also increased on the tumor cells in the glioblastoma biopsy samples, as compared to glial cells in the normal brain. Our results are consistent with those in the literature indicating that GRP78 expression is upregulated in tumors (reviewed in (15); (45,46). Two conditions frequently found in malignant tumors, hypoxia and hypoglycemia, are known to upregulate GRP78 expression (14,47). Recently, GRP78 expression has been associated with chemoresistance making it a promising target for cancer therapy (reviewed in 15); (45,48).

In summary, our data indicate that irradiation sensitizes primary human brain MvEC to the apoptosis-inducing effect of rK5 and that this pro-apoptotic signal requires LRP1 internalization of GRP78 and p38 MAP kinase activity. As the cell surface binding partner for rK5 (GRP78) and its scavenger receptor partner (LRP1) (49) are both expressed on MvEC in glioblastoma tumor biopsy samples, these data suggest that rK5 treatment post-irradiation should be considered in the design of new therapies for patients with glioblastoma tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

We thank Mrs. Rhonda Carr for assistance in preparing this manuscript and Dr. Fiona Hunter for critical review.

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Fig 1. Irradiation Sensitizes Primary Human Brain MvEC to rK5-Induced Apoptosis
A, Primary human brain MvEC plated on collagen type IV in M199 media with 10% FBS, 5 ng/ml bFGF and 10 ng/ml VEGF were treated with rK5 (or vehicle) for 17 h, and then subjected to a TUNEL assay. B–D, Primary human brain MvEC cultured as described above were irradiated, allowed to recover for 20 h, and then re-plated onto collagen-coated wells in fresh media (4 h), followed by treatment with rK5 or vehicle for 17 h or for the indicated times, and then subjected to a TUNEL assay (B) or detergent lysis, SDS-PAGE, and immunoblotting with the indicated antibodies (C&D). Tumor necrosis factor α (TNFα) induction of apoptosis was used as a positive control in the TUNEL assays.
Fig. 2. GRP78 Is Necessary for rK5-Induced Apoptosis of Human Brain MvEC
A, Unirradiated and irradiated primary human brain MvEC (plated and treated as described in the legend for Fig. 1) were surface biotinylated 24 h post-irradiation, detergent lysed, immunoprecipitated with anti-GRP78 antibody, and subjected to SDS-PAGE. B, Unirradiated primary human brain MvEC were treated with siRNA for 48 h, re-plated onto collagen-coated wells in fresh media, and subjected to a TUNEL assay or detergent lysed and immunoblotted with the indicated antibodies. C, Irradiated primary human brain MvEC were treated immediately with siRNA for 20 h, re-plated onto collagen-coated wells in fresh media, and treated with siRNA for an additional 4 h, and subjected to a TUNEL assay or detergent lysed and immunoblotted with the indicated antibodies. TNFα induction of apoptosis was used as a positive control in the TUNEL assays.
Fig. 3. LRP1 Is Necessary for rK5-Induced Apoptosis of Primary Human Brain MvEC

A&B. Irradiated (A) and unirradiated (B) primary human brain MvEC were treated with rK5 or vehicle (as described in the legend for Fig. 1) or rec-RAP and then subjected to TUNEL assay or detergent lysed and immunoblotted with the indicated antibodies. C. Primary human brain MvEC were irradiated and immediately treated with siRNA for 20 h, the cells re-plated and treated with siRNA for an additional 4 h, followed by treatment with rK5 or vehicle (17 h) and then subjected to a TUNEL assay. D, Primary human brain MvEC were treated with siRNA for 48 h, re-plated and treated with rK5 or vehicle (17 h) and then subjected to a TUNEL assay. TNFα induction of apoptosis was used as a positive control in the TUNEL assays.
Fig. 4. p38 MAP Kinase is Necessary for rK5-Induced Apoptosis of Irradiated Brain MvEC

A&B. Primary human brain MvEC were irradiated, allowed to recover, re-plated and treated with rK5 or vehicle for 17 h, or for the indicated time as described in the legend for Fig. 1, followed by detergent lysis, and immunoblotting with the indicated antibodies (A). Inhibitors were added 30 min prior to rK5 addition (B). C. Primary human brain MvEC were treated with siRNA for 48 h, irradiated, treated with siRNA for an additional 20 h, re-plated and treated with siRNA for 4 h then treated with rK5 or vehicle (17 h) followed by a TUNEL assay. TNFα induction of apoptosis was used as a positive control in the TUNEL assays.
Fig. 5. p38 MAP Kinase is Necessary for rK5-Induced Apoptosis of Unirradiated Brain MvEC

A. Primary human brain MvEC were plated and treated with rK5 or vehicle for 17 h, as described in the legend for Fig. 1, followed by detergent lysis, and blotting with the indicated antibodies, or subjected to TUNEL Assay. Inhibitors were added 30 min prior to rK5 addition.

B. Primary human brain MvEC were treated with siRNA for 68 h, re-plated and treated with siRNA for an additional 4 h and then treated with rK5 or vehicle (17 h), followed by a TUNEL assay. TNFα induction of apoptosis was used as a positive control in the TUNEL assays.
Fig. 6. Increased Expression of GRP78 in Glioblastoma Tumor Samples

A. Specific expression of GRP78 on brain MvEC and tumor cells in glioblastoma tumor biopsy samples. Sections of glioblastoma tumor (GBM) or normal brain (NB) were reacted with 15 µg/ml anti-GRP78 IgG, 10 µg/ml anti-vWf IgG, or 15 µg/ml IgG (20 h, 4°C), followed by an HRP-conjugated secondary IgG, substrate, and hematoxylin counterstaining. Arrows denote microvessels. The scale bar represents 20µm. Magnification – 400X. B. Immunoblotting of glioblastoma samples demonstrates increased GRP78 expression. Tissue samples were homogenized in detergent lysis buffer with protease inhibitors, and 100 µg of lysate subjected to 10% SDS-PAGE, followed by immunoblotting (data not shown). Bands were subjected to densitometry and the ratio of GRP78 to HSP70 and GRP78 to GAPDH determined and plotted as bar graphs (mean ± S.E.M) for the NB and GBM samples (B).