HARPΔ111-136 enhances radiation-induced apoptosis of U87MG glioblastoma by induction of the proapoptotic protein CHOP

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Abstract. We previously demonstrated, using the glioblastoma cell line U87MG as an experimental model, that the adenoviral mediated overexpression of the truncated protein HARPΔ111-136 inhibits the proliferation of these cells in vitro as well as tumor growth and angiogenesis in vivo. This study focused on identifying the underlying mechanisms for the observed antitumoral effect. The present study demonstrated that HARPΔ111-136 induced the ATF4/ATF3/CHOP cascade resulting in a strong expression of the proapoptotic protein CHOP, leading to tumor cell apoptosis as demonstrated by PARP cleavage and FACS analysis. siRNA-mediated CHOP gene silencing abolished Ad-HARPΔ111-136 induced apoptosis. Moreover, Ad-HARPΔ111-136 increased the expression of the death receptor DR5 and enhanced U87MG cells sensitivity in vitro to TRAIL a DR5 ligand with subsequent activation of caspase 8. Infection of U87MG cells with Ad-HARPΔ111-136 also enhanced radiation-induced apoptosis. In vivo, the combination of Ad-HARPΔ111-136 and radiation therapy resulted in a striking inhibition (92%) of the growth of U87MG xenografts, resulting from the potent effect on tumor angiogenesis and tumor cell apoptosis as determined by TUNEL analysis. Taken together, our results indicated that the inhibitor HARPΔ111-136 sensitized U87MG cells to apoptosis.

Introduction

Heparin affin regulatory peptide (HARP), also called pleiotrophin, is a secreted growth factor known for its oncogenic (1), mitogenic (2) and angiogenic activities (3,4). HARP has been shown to signal through two high affinity receptors, the receptor-type protein tyrosine phosphatase β/5 (RPTPβ/5) (5,6) and the anaplastic lymphoma kinase (ALK) (7). HARP together with its receptors are strongly overexpressed in several tumor types particularly glioblastoma (3,8-10). Glioblastoma multiforme (GBM) is the most aggressive and frequent adult primary brain tumor (11-13). Resistance to apoptosis is a well known feature of glioblastoma cells leading to a poor response to chemotherapy and radiation therapy (14). Since HARP has been considered as a growth and a survival factor for cancer cells (15,16), blocking the HARP pathway could be of particular interest.

HARPΔ111-136 is a truncated form of HARP lacking the last 26 amino-acids that acts as a dominant negative effector by heterodimerization with the wild-type HARP protein (17,18). In a recent study (19) we demonstrated using the human glioblastoma cell line U87MG as an experimental model, that overexpression of this truncated protein inhibited in vitro and in vivo proliferation of this tumor cell line as well as angiogenesis. A replication-defective adenovirus encoding this truncated protein (Ad-HARPΔ111-136) was constructed and our results showed that direct intra-tumoral administration of Ad-HARPΔ111-136 inhibited growth and angiogenesis of established U87MG xenografts in nude mice. However, the underlying mechanisms by which HARPΔ111-136 inhibits tumor proliferation remain to be explored. Herein we identified, in U87MG cells infected with Ad-HARPΔ111-136, the activation of a set of genes ATF4/ATF3/CHOP which are components of the unfolded protein response (UPR) pathway (20,21).

The UPR is primarily a survival response; however, in the case of intensive or persistent ER-stress, it triggers apoptosis resulting in cell death (22). The bZIP transcription factor C/EBP homologous protein (CHOP) is a critical effector of the ER stress proapoptotic arm. CHOP leads to cell cycle arrest, increases expression of a number of proapoptotic genes (20,21) and decreases expression of the anti apoptotic protein Bcl-2 (22,23). In the present study, we provide the first evidence of a proapoptotic effect of the inhibitor HARPΔ111-136 on the U87MG glioblastoma cell line. Ad-HARPΔ111-136 induced a strong expression of the protein CHOP leading to increased apoptosis of this highly malignant cell line (14). Furthermore,
a therapy combining a systemic Ad-HARPΔ111-136 administration with radiotherapy triggered massive apoptosis in established tumor xenografts in vivo.

Materials and methods

Cell culture. The human glioblastoma U87MG cell line (American Type Culture Collection, Illkirch, France) was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Cergy Pontoise, France). When specified, cells were irradiated at a dose of 10 Gy using a 250 kV X-Ray XSD 225 Comet tube, operating at 200 kV and 15 mA with a 0.2 mm Cu-filter. This dose was chosen based on a preliminary multiple dose-response experiment.

Virus description and infection. Ad-HARPΔ111-136 an E1-E3-deleted recombinant adenovirus that expresses the first 111 amino acids of the protein HARP was previously described (19). Adenovirus expressing no transgene (Ad-C01) was used as control. Ad-CMV-GFP is an adenovirus expressing the green fluorescent protein (GFP), Ad-K1-5 is an adenovirus expressing the five kringles of the angiostatin (24). Adenovirus infection was carried out at a multiplicity of infection (MOI) of 500 PFU/cell, in culture medium supplemented with 2% FBS.

Reverse transcription polymerase chain reaction. Reverse transcription was performed from 1 μg total RNA, using Thermoscript RT-PCR system (Invitrogen) with oligo dT primer. The primers used for polymerase chain reaction are ATF3: sense 5’TGATGCTTCAACACCCAGGCC3’, reverse 5’AGGGGCCGATGGCAGAAGCA3’; ATF4: sense 5’AGT

Heparin acryl beads. Heparin-acryl beads (H5263, Sigma), 500 μl, were washed 5 times in PBS and incubated overnight at 4°C with 1 ml of mice plasma. After 5 washes with PBS, the beads were boiled in 100 μl of Western blot analysis sample buffer and resolved on SDS-PAGE.

Animals. Experiments were conducted in accordance with the recommendations of the National Institutes of Health for animal experimentation. Female NMRI nude mice (Janvier, Le Genest sur Isle, France) were used. When specified, mice received an intravenous injection of 5x10⁹ PFU of Ad-C01 or Ad-HARPΔ111-136 in 100 μl PBS. When specified, mice received three X-ray irradiations, at 3 days intervals, delivered at a dose of 2.5 Gy, using a 250 kV X-Ray XSD 225 Comet tube operating at 200 kV and 15 mA with a 0.2 mm Cu-filter.

Tumor cell inoculation in vivo. U87MG, 2x10⁶ cells in 100 μl, were injected subcutaneously into the back of nude mice. When mean tumor volumes reached 66±20 mm³ mice were randomly assigned to each treatment group. The tumor size was monitored every two or three days by measuring the shortest (a) and the longest (b) tumor diameters with a dial caliper (b perpendicular to a) and the tumor volume was calculated as [(a + b/2)³ x π/6] (20). Blood samples were collected on sodium citrate and plasma were incubated 30 min at 56°C to inactivate residual viruses.

Immunostaining. Immunohistochemistry was performed as previously described (25,26).

Statistics. Data were analyzed using the unpaired Student’s t-test. All experiments were repeated independently at least twice. A P<0.05 was considered to be statistically significant.
Results

Ad-HARPΔ111-136 activates ER stress signaling in glioblastoma U87MG cells. The deleted HARP peptide HARPΔ111-136 was expressed in cells using an adenovirus as described previously (19). RT-PCR was performed on RNA transcripts extracted from U87MG cells infected for 48 h with Ad-HARPΔ111-136 or the control Ad-CO1 virus. Results (Fig. 1A) show an increase in mRNA levels of DDIT3 (CHOP), CREB-2 (ATF4) and ATF3 genes. A rise in the levels of the proteins (Fig. 1B) ATF4, ATF3 and CHOP was demonstrated by Western blot analysis after Ad-HARPΔ111-136 treatment.

The increase in transcript and protein levels of CREB-2 (ATF4) and DDIT3 (CHOP) in Ad-HARPΔ111-136-infected U87MG cells suggests the activation of the protein kinase-like ER kinase (PERK) pathway of the ER stress signaling. Therefore, we investigated the effect of Ad-HARPΔ111-136 on the other pathways involved in ER stress signaling (20,21). No change in expression was seen for ATF6 gene involved in the first pathway (data not shown). The proteins JNK, P38 MAPK were phosphorylated (Fig. 1C) after Ad-HARPΔ111-136 infection, suggesting the activation also of the third inositol-requiring kinase 1 (IRE1) pathway.

Ad-HARPΔ111-136 increases expression of CHOP and apoptosis in U87MG cells. The effects of Ad-HARPΔ111-136 on the expression of the protein CHOP in U87MG cells were assessed by Western blotting. Our results showed that the 30 kDa CHOP protein was strongly expressed 24 h after the Ad-HARPΔ111-136 infection (not shown) and maintained for up to 72 h (Fig. 2A), whereas no signal could be detected in cell lysates derived from control Ad-CO1 infected cells.

CHOP has been described as a proapoptotic protein (27,28) thus we examined if CHOP expression was accompanied by an increase in apoptosis of Ad-HARPΔ111-136-infected U87MG cells. Apoptosis was assessed by Western blotting using an antibody directed against the cleaved form of poly (ADP-ribose) polymerase 1 (PARP-1), the main substrate of caspase 3. As shown in Fig. 2A, cleaved PARP was found in cells infected with Ad-HARPΔ111-136 and not in control Ad-CO1-infected cells indicating that Ad-HARPΔ111-136 expression increased apoptosis.

It is known that HARP activates protein kinase C (PKC) through receptor RPTPB/Z signaling pathway (29) and it has been shown that inhibition of PKC leads to increased expression of CHOP via β-catenin accumulation (30). Therefore we monitored PKC phosphorylation after Ad-HARPΔ111-136 infection and demonstrated (Fig. 2C) that as expected over-expression of the HARP peptide HARPΔ111-136 inhibited PKC phosphorylation in U87MG cells.

In order to confirm that ER stress induction is mediated by the expression of the peptide HARPΔ111-136 and not by the forced expression of any protein in the cell, two additional controls were performed, using viruses expressing either the green fluorescent protein (Ad-GFP) or the five kringles of angiostatin Ad-(K1-5) (24). The virus Ad-(K1-5) encodes a protein which, like the peptide HARPΔ111-136 used in the present study, is a truncated and secreted protein. The absence of the expression of CHOP (Fig. 2B) in U87MG cell lysates infected with Ad-GFP or Ad-(K1-5) indicates that the observed upsurge in CHOP expression is related to the peptide HARPΔ111-136 and not to the over production of any other transgene after the viral infection.

Ad-HARPΔ111-136 sensitizes U87MG cells to TRAIL-induced apoptosis. CHOP is a well-documented regulator of death receptor 5 (DR5) gene expression (31). Thus we investigated the putative role of DR5 receptor in Ad-HARPΔ111-136 induced apoptosis. Cell lysates and mRNA were prepared from Ad-HARPΔ111-136 or Ad-CO1 U87MG infected cells. The RT-PCR analysis (Fig. 3A) revealed that DR5 mRNA expression was increased in cells infected by Ad-HARPΔ111-136. Western blotting demonstrated an increased DR5 protein expression (Fig. 3B) in U87MG cells infected with Ad-HARPΔ111-136. This effect was accompanied by a considerable increase of caspase-8 cleavage, a downstream effector of DR5-induced apoptosis (Fig. 3B).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a DR5 death receptor ligand. As we demonstrated that Ad-HARPΔ111-136 infection increased DR5 expression while control virus did not, we speculated that Ad-HARPΔ11-
136 would enhance TRAIL-induced U87MG cell apoptosis. To verify this hypothesis, U87MG cells infected with Ad-HARPΔ111-136 or control Ad-CO1 for 48 h were treated for 30 h with recombinant TRAIL at a final concentration of 100 ng/ml. As shown in Fig. 3C, TRAIL induced apoptosis in only 0.67% of Ad-CO1-infected cells; this demonstrates the magnitude of resistance of U87MG cells to apoptosis. In Ad-HARPΔ111-136 infected cells, TRAIL-induced apoptosis was significantly enhanced (p<0.001) by 5-fold compared to Ad-CO1 infected cells (Fig. 3C). Thus, Ad-HARPΔ111-136 infection sensitizes U87MG cell line to TRAIL-induced apoptosis by increasing the expression of the DR5 receptor.

Ad-HARPΔ111-136 enhances the radio-sensitivity of U87MG tumor cells. U87MG are known to be radiation-resistant cells, thus experiments were designed to evaluate whether the
expression of the proapoptotic protein CHOP via Ad-HARPΔ111-136 could modify radiation-induced apoptosis in U87MG cells. Cultured U87MG cells were irradiated at a dose of 10 Gy and infected with control Ad-CO1 or Ad-HARPΔ111-136 viruses. After 96 h, cells were stained with propidium iodide and apoptosis was quantified by measuring the sub-G1 population by FACS. A positive control for apoptosis was prepared by treating U87MG with the therapeutic agent temozolomide (TMZ) and demonstrated the extent of resistance of this cell line to apoptosis. Values represent the mean of four experiments ± SD. *** p<0.001 Student's t-test.

CHOP is involved in the apoptotic response of U87MG to Ad-HARPΔ111-136 and radiation. In order to determine whether CHOP contributes to Ad-HARPΔ111-136-induced cell death, siRNAs were used to abolish CHOP expression after Ad-HARPΔ111-136 infection. As expected, CHOP siRNA mediated efficient knockdown of CHOP protein expression in cells infected with Ad-HARPΔ111-136 (Fig. 5A). No effect was observed with non-relevant siRNA. The effect of CHOP siRNA on the apoptosis (sub-G1 population) induced by Ad-HARPΔ111-136 infection was estimated by FACS analysis. As shown in Fig. 5B no changes in the sub-G1 population were detected in Ad-CO1 infected cells after CHOP knockdown. The use of CHOP siRNA in Ad-HARPΔ111-136-infected cells significantly decreased (47%, p<0.001) the number of cells in the sub-G1 phase when compared with the use of non-relevant siRNA. A similar drastic and significant decline (p<0.001) of apoptosis was observed after knockdown of CHOP when irradiation was combined with Ad-HARPΔ111-136 treatment. These results collectively indicate that the knockdown of CHOP in U87MG cells decreased the Ad-HARPΔ111-136 induced apoptosis. Taken together these results indicate that CHOP directly contributes to Ad-HARPΔ111-136 induced apoptosis.

Ad-HARPΔ111-136 therapy enhances the growth inhibition effect of radiation therapy on established U87MG xenografts.
in vivo. To assess the in vivo effect of a bitherapy combining Ad-HARPΔ111-136 systemic injection with radiation therapy (RT), two million U87MG cells were subcutaneously injected into the back of nude mice. When mean tumor volumes reached 66±20 mm$^3$, mice were randomized into five groups - Ad-CO1, Ad-HARPΔ111-136, RT, RT + Ad-CO1 and RT + Ad-HARPΔ111-136 (bi-therapy), and treatments were initiated. Three total body irradiations were performed on RT groups at a dose of 2.5 Gy at two days interval, starting on day 13 after tumor inoculation. Viruses were injected intravenously (5x10$^9$ PFU) immediately after the last irradiation.

Our results demonstrated that on day 26, U87MG mean tumor volume (Fig. 6A) was significantly lower (p<0.01) in all groups that received a therapy compared to Ad-CO1 control group. There was no difference between groups receiving RT alone or RT + Ad-CO1 indicating that the viral infection itself had no influence on the effect of the radiation therapy. In accordance with our previous data using intra-tumoral Ad-HARPΔ111-136 injection (19), a single systemic injection of Ad-HARPΔ111-136 inhibited tumor growth by 40% (p<0.01) in all groups that received a therapy compared to Ad-CO1 control group. There was no difference between groups receiving RT alone or RT + Ad-CO1 indicating that the viral infection itself had no influence on the effect of the radiation therapy. In accordance with our previous data using intra-tumoral Ad-HARPΔ111-136 injection (19), a single systemic injection of Ad-HARPΔ111-136 inhibited tumor growth by 40% (p<0.01) when compared to control virus. A limited tumor growth was observed with the combination therapy, Ad-HARPΔ111-136 + RT. Furthermore, the bitherapy was significantly more efficient than each monotherapy (RT CO1 vs. RT+Ad-HARPΔ111-136: p<0.05 on days 26 and 28, p<0.01 on day 30; Ad-HARPΔ111-136 vs. RT+Ad-HARPΔ111-136: p<0.001 on days 26, 28 and 30).

After systemic injection it is known that adenovirus infects the liver. To assess the in vivo peptide HARPΔ111-136 production after the viral infection, few mice were sacrificed during the first week after infection, their blood collected and their livers excised, fixed and processed for immunohistochemistry. The in vivo HARPΔ111-136 production was confirmed by anti-HARP immunostaining of liver sections as shown in Fig. 6B. Hematoxylin-eosin-safran staining of liver sections of all groups revealed a slight anisonucleosis related to a regeneration process without cytolysis.

Circulating HARPΔ111-136 in mice was concentrated from sera on heparin-acrylic beads and in vivo HARPΔ111-136 production was assessed by Western blot analysis using anti-HARP antibodies (Fig. 6C). The 15 kDa protein (HARPΔ111-136) appeared in mouse sera four days after Ad-HARPΔ111-136 infection, and its level dramatically increased 8 days after the infection indicating a strong production of the HARPΔ111-136 peptide in vivo.

The combination of RT and Ad-HARPΔ111-136 treatments reduces tumor vessel density and triggers massive apoptosis of U87MG tumor cells in vivo. At the end of the experiment described before, U87MG tumors were excised and processed for immunohistological analysis. Tumor vascularization was assessed using anti-CD31 antibody (Fig. 7A). Large and thick tumor blood vessels, a feature of this highly angiogenic tumor, were only present in the Ad-CO1 control tumor.
sections, whereas blood vessels in the Ad-HARPΔ111-136 group were much smaller in size and thinner. The normalized profile of blood vessels was constantly observed with the combination RT + Ad-HARPΔ111-136 therapy. The slides were scanned and the staining quantified using the automated PIXCYT software (26). The vascular density was significantly reduced (30%, p<0.001) in the group subjected to bitherapy when compared to RT + Ad-CO1, indicating that Ad-HARPΔ111-136 treatment counteracted the angiogenic effect of radiation therapy (32).

Apoptotic cells present in tumor sections were visualized in situ using TUNEL analysis. When compared with the Ad-CO1 control group (Fig. 7B), a single treatment by Ad-HARPΔ111-136 or RT significantly increased tumor U87MG cell apoptosis (Fig. 7C) by 6-fold (p<0.01) and 4.5-fold (p<0.05) respectively, while the combination of RT + Ad-HARPΔ111-136 increased apoptosis by more than 13-fold (p<0.001%) compared to RT + Ad-CO1. The strong intensity of tumor apoptosis after the double treatment is illustrated by the background red color of the slides which was observed only in this group. These findings confirmed in vivo that the peptide HARPΔ111-136 sensitized U87MG cells to apoptosis.

Discussion

HARP has been described as a growth factor for several tumor types (3,15,33) and in a previous study using U87MG glioblastoma cell line as an experimental model, we demonstrated that the HARP inhibitor HARPΔ111-136 exerts antiproliferative and potent anti-angiogenic activities. The aim of the present study was to investigate the mechanism of HARPΔ111-136 biological action; a recombinant adenovirus was used to produce high levels of the inhibitor HARPΔ111-136 in the glioblastoma U87MG cell line. Our results using RT-PCR and Western blotting demonstrated that the Ad-HARPΔ111-136 activated the ATF4/ATF3/CHOP pathway which is characteristic of the UPR response to ER stress. The ER stress involves three signaling pathways initiated by ATF6, PERK and IRE1 (21). Here we demonstrated the activation of two of these pathways in U87MG cells after infection with Ad-HARPΔ111-136. The increase in mRNA expression of ATF4 (CREB-2) and DDIT3 (CHOP) genes confirmed the activation of the PERK pathway. The activation of the IRE1 pathway was supported by the observed phosphorylation of JNK and P38 MAPK after Ad-HARPΔ111-136 infection.
We focused our interest on the DDIT3 gene encoding the protein CHOP which was absent in steady state U87MG cells and was highly induced at mRNA and protein levels after Ad-HARPΔ111-136 treatment. The ER facilitates the intracellular trafficking of membrane and secreted proteins, however, when the capacity of the system is exceeded, the UPR is activated and CHOP is produced. Thus, in our experimental model CHOP could be simply induced by the protein overexpression after the viral infection. In order to verify this hypothesis U87MG cells were infected by recombinant adenovirus encoding either unsecreted GFP or a truncated form of angiotatin (K1-5). Like HARPΔ111-136, K1-5, is a truncated and secreted protein. In both cases CHOP was not induced, thus, our results are consistent with a specific effect of HARPΔ111-136 on CHOP expression.

How HARPΔ111-136 activates ER stress is an important question that arises. We previously demonstrated that HARPΔ111-136 acts as a dominant negative effector of HARP by heterodimerizing with the wild-type HARP (17,18). We hypothesized that expression of HARPΔ111-136 may indirectly lead to ER stress by preventing: i) HARP-induced oligomerization of the receptor RPTPβ and, ii) the downstream activation of protein kinase C (PKC) (29). This report demonstrated that Ad-HARPΔ111-136 reduced phosphorylation of PKC in U87MG cells. It is known from the literature that inhibition of PKC (30) leads to the rapid accumulation of β-catenin that activates early ER stress signaling via CHOP. Thus, we speculate that this mechanism is involved in HARPΔ111-136 effect. However, the interaction of the peptide HARPΔ111-136 with another intracellular pathway is not excluded and remains to be investigated.

Several studies suggest a potential role for CHOP in ER stress-induced neuronal cells death (27,28,34). In the present study, the expression of CHOP after the Ad-HARPΔ111-136 infection was accompanied by a 10-fold increase of apoptosis in U87MG cells. Apoptosis was confirmed by the activation of caspase 3 through the cleavage of its main substrate PARP. Furthermore, our in vitro study demonstrated that knockdown of CHOP using RNA interference considerably reduced the HARPΔ111-136-induced apoptosis, indicating a crucial role for CHOP in the downstream apoptotic effects of HARPΔ111-136. Our findings indicating that interruption of the HARP stimulation may lead cells to apoptosis are consistent with studies by Powers et al (33) who reported that targeting the HARP pathway in U87MG cells, using a ribozyme abolishing ALK expression, a HARP receptor, resulted in an increased apoptosis in U87MG xenografts. Other studies by Grzelinski et al (16) showed that knockdown of HARP using ribozymes induced apoptosis in U87MG cells.

CHOP is one of the critical effectors of the ER stress proapoptotic arm and activates the transcription of several genes that encode proapoptotic factors (35,36). Our study demonstrated that infection of U87MG by Ad-HARPΔ111-136 resulted in an increased expression of the death receptor DR5 a downstream target of CHOP. It is known that upon activation by its ligand TRAIL, the death receptor DR5 mediates a potent proapoptotic effect (37,38). The results of the present study demonstrated the extreme resistance of U87MG cells to TRAIL-induced cell death, confirming observations by others (39). Our results demonstrated that Ad-HARPΔ111-136 enhanced U87MG cell sensitivity to TRAIL-induced apoptosis, through up regulation of its receptor DR5 and subsequent cleavage of the downstream caspase 8. These findings are in agreement with recent studies demonstrating that combining TRAIL with inducers of ER stress resulted in a strong sensitization to apoptosis of tumor cell lines (40) including several glioma cell lines (41,42).

It is noteworthy that the adenoviral E1 and E3 genes encode proteins that specifically counteract apoptosis induced by death receptors (43). Despite deletion of these genes in our adenoviral construction, TRAIL-induced cell death was always higher in non-infected U87MG cells than in control Ad-CO1-infected cells (data not shown). Thus, in our study, the magnitude of the HARPΔ111-136 enhancing effect on TRAIL-induced apoptosis may have been underestimated.

Another way to induce tumor cell apoptosis is irradiation. Our data provide evidence, for the first time, that the peptide HARPΔ111-136 enhanced and even synergized in vitro with the effect of radiation therapy in U87MG cell line, through increased apoptosis of the tumor cells as analyzed by flow cytometry. This effect is CHOP-dependent as demonstrated by siRNA studies.

In our previous study (19) the anti-proliferative and antiangiogenic activities of HARPΔ111-136 were demonstrated in vivo on established subcutaneous glioblastoma U87MG tumors in which a single intra-tumoral injection of Ad-HARPΔ111-136 inhibited U87MG tumor growth by 45% and reduced tumor vessel density. In the present study, a single systemic Ad-HARPΔ111-136 injection in vivo also inhibited angiogenesis in subcutaneous glioblastoma U87MG tumors thus confirming the findings with intra-tumor injection. In the present study, the therapeutic molecule HARPΔ111-136 was produced in the liver causing minimal damage to hepatocytes as shown by the immunohistochemical analysis of liver sections, the molecule was drained into the blood as shown by Western blotting and finally acted distantly on the tumor. In vivo, TUNEL analysis of tumor sections provided evidence that the systemic Ad-HARPΔ111-136 administration resulted in an increased tumor apoptosis and that, as illustrated in Fig. 7B, the combination of Ad-HARPΔ111-136 and radiation therapy (bitherapy) induced a massive tumor apoptosis.

In vivo tumor growth was strikingly inhibited during one month in the group subjected to bitherapy (Ad-HARPΔ111-136 + RT) owing to both antiangiogenic and proapoptotic effects as demonstrated by immunohistochemical studies. Statistical studies (Student’s t-test) indicated that the bitherapy was significantly more efficient in reducing tumor growth than each monotherapy. Our results obtained with Ad-HARPΔ111-136 and irradiation are coherent with those of Yacoub et al (44) who reported in vitro radiosensitization of primary human glioblastoma cells with OSU-03012, a molecule known to induce the ER stress. Moreover, studies by Hideshima et al (45) showed that bortezomib, that induces ER stress, can restore the sensitivity of myeloma cells to chemotheraphy induced apoptosis. As the peptide HARPΔ111-136 sensitizes U87MG cells to the effects of both irradiation and TRAIL treatment in vitro, the combination of the three agents together may result in greater cell death and a tight control of tumors.
In conclusion, our data emphasize the importance of CHOP signaling in apoptosis induced by the HARP inhibitor HARPΔ111-136. There is ample evidence showing that the peptide HARPΔ111-136 is a potent anti-angiogenic agent. Herein, we demonstrated a novel proapoptotic mechanism of action of this peptide on a glioblastoma cell line. Thus, due to its dual effect, inhibiting angiogenesis and, simultaneously, promoting apoptosis of the tumor cells, the peptide HARPΔ111-136 appears to be a promising multi-targeted pharmacological tool. Unlike glioblastoma, normal adult brain tissues do not express HARP and its two receptors (10), thus, tumor cells would be more susceptible to the HARPΔ111-136 cytotoxic effect than normal cells. A hallmark feature of U87MG cell line is an intense resistance to stimuli inducing apoptosis, therefore, HARPΔ111-136 could be exploited as a tool to sensitize cancer cells to standard therapy, with minimal potential toxicity.

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