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ORIGINAL ARTICLE

The Role of Glycyrrhizin, an Inhibitor of HMGB1 Protein, in Anticancer Therapy

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Abstract Certain anticancer drugs, such as the peptide CAMEL (aa sequence KWKLFKKIGAULKVL) induce necrotic type of cell death. During this process, a protein termed high mobility group box 1 (HMGB1) is released from cell nucleus into cytoplasm and then into extracellular milieu. Outside of cells, it becomes a proinflammatory cytokine. Its effects range from stimulation of cancer as well as endothelial cell proliferation, to activation of angiogenesis, cell motility and induction of inflammatory conditions. Release of HMGB1 cytokine during the course of anticancer therapy has negative effects upon the therapy itself, since it leads to tumor relapse. We assumed that the inhibition of HMGB1 activity may be conducive towards better therapeutic results in case of drugs inducing necrotic cell death. In this context we studied glycyrrhizin (GR), a triterpenoid saponin glycoside of glycyrrhizic acid and a well-known inhibitor of HMGB1. We have shown that GR inhibits proliferation and migration of cells stimulated by HMGB1 cytokine, as well as HMGB1-induced formation of blood vessels and reduces inflammatory condition (lowering tumor necrosis factor α levels). GR-mediated inhibition of HMGB1 activity (CAMEL-induced release) impedes, in turn, tumor regrowth in mice. As expected, inhibited tumor regrowth is linked to diminished tumor levels of the released HMGB1 and reduced inflammatory condition. To conclude, the use of GR significantly improved anticancer effectiveness of the CAMEL peptide.

Keywords Anticancer peptide · Glycyrrhizin · HMGB1 · Necrosis · Tumor therapy

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Introduction

Certain peptides are among the most intensely studied anticancer drugs. Anticancer peptides present benefits including ease of synthesis, high degree of cancer tissue penetration and low immunogenicity. Their disadvantage is a relatively short lifetime in circulation (Smolarczyk et al. 2009). An example of these peptides is CAMEL (aa sequence: KWKLFKKIGAVLKVL) (Smolarczyk et al. 2010). CAMEL induces necrotic cell death accompanied by release of several proteins from cell including high mobility group box 1 (HMGB1) protein. Inside cells, this protein is tightly associated with cell nucleus where it stabilizes chromatin conformation, and takes part in regulation of gene expression and DNA repair (Lotze and Tracey 2005; Scaffidi et al. 2002; Sims et al. 2010; Tang et al. 2010).

HMGB1 is released during necrosis from cell nucleus into cytoplasm and into extracellular space (Gauley and Pisetsky 2009; Rovere-Querini et al. 2004). HMGB1 release during necrosis is passive (Raucci et al. 2007). A mechanism of active HMGB1 release from cells has also been described (Klune et al. 2008). Released HMGB1 is responsible for increased cell proliferation (Palumbo et al.

2004), activation of angiogenesis (Mitola et al. 2006; Schlueter et al. 2005), increased cell motility, and induction of inflammatory conditions (Srikrishna and Freeze 2009; Yang et al. 2005). All of these processes are conducive towards tumor relapse (Campana et al. 2008). According to Tang et al. (2010), HMGB1 is a protein the overexpression of which is linked to the following "hallmarks of cancer": unlimited proliferation ability, angiogenesis, resistance to apoptosis, production of cells' own growth factors, lack of susceptibility to growth inhibitors, inflammatory state, and metastasizing (Hanahan and Weinberg 2011).

Thus, inhibition of HMGB1 activity during therapy with drugs inducing necrotic cell death may positively affect antitumor therapy. Inhibitors of HMGB1 protein include specific antibodies, box A domain of HMGB1, or low-molecular weight compounds such as glycyrrhizin (GR) (Girard 2007; Zhu et al. 2010). The latter group of inhibitors affects HMGB1 activity following extracellular release. Another group of inhibitors involves agents that block protein release from cells. This latter group includes ethyl pyruvate, acetylcholine, nicotine or stearoyl lysophosphatidylcholine (Ellerman et al. 2007; Yang and Tracey 2010).

On this basis, we raised the possibility that inhibition of HMGB1 activity and its release from cells can improve therapeutic effectiveness of drugs inducing necrotic death of cancer cells. Therefore, we aimed to investigate the effect of GR, an inhibitor of HMGB1, upon the growth kinetics of experimental melanoma B16-F10 tumors in mice treated with CAMEL.

Materials and Methods

CAMEL Peptide Synthesis

CAMEL (aa sequence KWKLFKKIGAVLKVL-NH_2) was synthesized as described elsewhere (Smolarczyk et al. 2010).

Identification of HMGB1 Protein in Cells and in Culture Medium Conditioned by B16-F10 Cells that Underwent Necrotic Death

B16-F10 cells were cultured in RPMI1640 medium supplemented with 10 % bovine serum. The medium was then replaced with serum-free medium and CAMEL peptide (final concentration: 10 μM) was added. After 3 h the medium from such cultures was collected and passed through 10 kDa molecular weight cut-off concentrators. The cultured cells were lysed using RIPA buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate). The concentrated samples were separated electrophoretically (using polyacrylamide gels) and transferred onto nitrocellulose membranes. The membrane was incubated with anti-HMGB1 antibody (Abcam, UK; 0.5 μg/mL), and then with secondary anti-rabbit antibody (Vector Laboratories, USA) conjugated to horseradish peroxidase (HRP) allowing chemiluminescent detection. As positive control, cells were used that had been subjected to a triple freeze/thaw procedure, whereas negative control involved untreated cultured cells.

Effect of HMGB1 and GR on the Proliferation of Endothelial Cells

Bovine aortic endothelial cells (BAEC) (seeded at 7×10^3 cells/well) were cultured in 96-well plates containing four different concentrations (0.5, 1.0, 5.0 and 10 nM) of HMGB1 (Sigma-Aldrich, USA) and GR at 100 μM (Sigma-Aldrich, USA). As positive control, cells treated with fetal bovine serum-supplemented medium were used. As negative control, cells without HMGB1 added to the culture medium were used. After 24 h cell count was performed in each well. The experiment was repeated three times. The same experiment was done on bEND.3, Heca10 murine endothelial cells and NIH3T3 murine fibroblasts.

Effect of HMGB1 and GR upon Migration of NIH3T3 Cells

NIH3T3 cells (2×10^4) were placed in the upper compartment of the Boyden chamber containing serum-free medium. These cells were used since they were shown to have their motility unaltered by GR itself when no chemoattractant is present (Mollica et al. 2007). The lower compartments contained either serum-free RPMI1640 medium (negative control) or medium with chemoattractant, 25 nM HMGB1. A chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP; 2.5 mM) served as positive control. In order to check the effect of GR upon the ability of cells to migrate, various amounts of GR were added to the upper and lower compartments (final concentrations: 25, 50 or 100 μM). After 4 h inserts were removed, cells were fixed with 4 % paraformaldehyde and Giemsa staining was performed. Migrating cells were counted in five different fields and the results averaged. The experiment was repeated three times.

The Effect of HMGB1 and GR upon Microvessel Formation in Rat Aortic Rings

Aortic rings were obtained from 6-week-old male Fisher 344 rats, using a previously described procedure (Nicosia

and Ottinetti 1990). Animals were sacrificed using carbon dioxide. Following dissection, aorta was cut into rings about 1 mm high which were rinsed ten times with serum-free MCDB 131 medium (Sigma-Aldrich, USA) and placed in VI-A type agarose (Sigma-Aldrich, USA) prepared as follows. A sterile 1.5 % agarose solution was poured into 100 × 15 mm plates and left to solidify. Using two concentric rings (10 and 17 mm diameter) wells were cut out in the agarose layer. The wells were filled with phosphate buffered saline (PBS) solution containing fibrinogen (3 mg/mL; Sigma-Aldrich, USA). Next, 2.8 μL of a thrombin solution (Sigma T9549, activity: 1,506 NIH units/mL) was added per each milliliter of fibrinogen solution. Fibrin gel formed within 30 s; during this time aortic rings placed in the center of wells were embedded in the fibrin gel. After removal of agarose wells, the fibrin blocks containing aortic rings were placed in serum-free MCDB 131 medium using four-well culture plates. The cultures were maintained for 1 week at 37 °C/5 % CO_2/95 % air. In order to preserve fibrin-gel structure, the medium was supplemented with aminocaproic acid (300 μg/mL; Sigma-Aldrich, USA). After establishing of the culture, aortic rings were tested for their ability to form microvessels in the presence of daily-added HMGB1 (10 nM) and GR (100 μM). Forming microvessels were observed using an inverted phase-contrast microscope (Olympus IX71). Every group included 3–4 aortic rings. The experiment was performed twice.

Effect of CAMEL and GR upon Inflammatory Condition

Mice harboring B16-F10 melanoma tumors received intratumoral injections of CAMEL. Appropriate groups of animals received also an intraperitoneal injection of GR (2 mg in 400 μL PBS per animal). After 72 h blood was collected and coagulated, following which serum was isolated and stored frozen. The level of tumor necrosis factor (TNF)-α (inflammatory state indicator) was assessed in samples using a TNF-α ELISA kit (BD) according to the manufacturer's protocol. Amount of TNF-α (pg protein/1 mL) was determined. Results are mean of eight mice in each group.

Effect of CAMEL and GR (an HMGB1 Inhibitor) upon Growth of B16-F10 Tumors

C57BL/6 mice were inoculated with 2×10^5 B16-F10 melanoma cells. On 6th and 7th day following inoculation, CAMEL (100 μg/mL; green arrows) was injected intratumorally and GR (2 mg/animal) (blue arrows) was administered intraperitoneally. During the following 4 days GR only (2 mg/mouse/day) was administered. Tumor size was measured in two dimensions and inhibition of tumor growth monitored. The experiment was repeated twice. Every group contained at least five mice. Mice with developed B16-F10 tumors (6th day following inoculation) received intratumoral injections of CAMEL (100 μg/mL), accompanied by intraperitoneal administration of GR 2 mg/400 μL PBS/animal. During the following 4 days GR only (2 mg/mouse) was administered. Finally, tumors were excised, fixed and paraffin-embedded. Then, the material was cut into 5 μm-thick sections and stained with hematoxylin and eosin, anti-HMGB1 antibody (Abcam, UK), and F4/80 antibody (Abcam, UK) directed against macrophages. On the 15th day of therapy tumors were photographed. Hematoxylin and eosin (H&E)-stained specimens were photographed with 10× objective lens; anti-HMGB-1 antibody-stained specimens, and anti-macrophage antibody-stained specimens were photographed using 40× objective lens. The experiment was repeated three times. Three animals were included per group, and three to four sections, obtained from each mouse, were analyzed.

Immunohistochemical Identification of HMGB1

Paraffin-embedded B16-F10 tumor sections were deparaffinized, hydrated, and incubated in 0.3 % H_2O_2. Antigen retrieval was achieved by boiling in citrate buffer (10 mM; pH 6.0), whereas nonspecific binding was blocked with 2.5 % horse serum. The sections were incubated at room temperature for 1 h with rabbit anti-HMGB1 primary antibody (0.7 mg/mL; diluted 1:100, Abcam®) and with horse anti-rabbit secondary antibody conjugated to HRP (EC 1.11.1.7) from ImmPRESS™ REAGENT Anti-Rabbit Ig kit (Vector, USA). In order to obtain the final colored product the preparations were incubated with 3,3′-diaminobenzidine (DAB) from ImmPACT™ DAB kit. The enzymatic reaction yielded a brown-colored product. The preparations were then dehydrated and mounted with VectaMount™ medium. Three animals were included per group, and three to four sections, obtained from each mouse, were analyzed.

Identification of Macrophages

Paraffin-embedded B16-F10 tumor sections were deparaffinized and hydrated and then incubated in 0.3 % H_2O_2. Antigen retrieval was achieved by boiling in citrate buffer (10 mM; pH 6.0), whereas nonspecific binding was blocked with 10 % rabbit serum. The sections were then incubated at room temperature for 1 h with rat anti-mouse F4/80 primary antibody (diluted 1:150, AbD Serotec) and with reagents from Vectastain® ABC Kit (Vector, USA): rabbit anti-rat biotinylated secondary antibody (diluted

1:150), 1:1 mix of avidin and biotinylated HRP (EC 1.11.1.7; diluted 1:50). The color was developed with DAB (see above). The preparations were then dehydrated and mounted with VectaMount™ medium. Three animals were included per group, and three to four sections, obtained from each mouse, were analyzed.

Statistics

Results were analyzed using Statistica 6.0 software by a ANOVA. Student t test was used to analyze statistical differences between two analysed groups. Results were judged significant when $p < 0.05$.

Results

Necrotic Death is Associated with HMGB1 Release

B16-F10 cells cultured in RPMI1640 supplemented with 10 % bovine serum were switched to serum-free medium containing CAMEL (10 μM). After 3-h exposure, HMGB1 level was determined in the medium and cell lysates using Western blotting. HMGB1 was identified electrophoretically as the 30 kDa-migrating band. Control cells (necrotic death) were obtained by performing three cycles of freeze/thaw procedure. Untreated cells were used as negative control. HMGB1 protein was released from cells into the medium during freeze/thaw-induced necrosis, as well as following CAMEL exposure (Fig. 1). No such effect was observed in negative control. The constitutive level of HMGB1 in cells was shown in lysates. There was a decrease of HMGB1 in the necrosis and CAMEL groups, as compared with control group.

HMGB1 Increases Proliferation of Endothelial Cells while GR Abrogates this Effect

Bovine aortic endothelial cells (ATCC) were cultured using 96-well plates (3×10^3 cells/well) with serum-free medium containing various amounts of HMGB1 (0.5, 1, 5 and 10 nM). This medium contained also GR (100 μM). After 24 h HMGB1 increased proliferation of endothelial cells (Fig. 2). The highest increase in cell number was noted after addition of serum. Cell proliferation following HMGB1 addition was observed at each tested concentration of this protein. There were no differences in cell proliferation between different HMGB1 concentrations. Number of cells after incubation with different concentrations of HMGB1 was about 50 % more than the number of cells cultured with serum-free medium. Addition of GR, a HMGB1 inhibitor, inhibited proliferation yielding a number of cells similar to that in control culture without serum. The differences in cell number between HMGB1-treated group and HMGB1 + GR-treated group were statistically significant ($p < 0.05$). GR alone had no effect on cell proliferation. The same experiment was performed using bEND.3, Heca10 mouse endothelial cells and NIH3T3 murine fibroblasts. There was not any stimulatory effect on NIH3T3 fibroblasts, nor Heca10 mouse endothelial cells, and a negligible effect on bEND.3 endothelial cells (data not shown).

Glycyrrhizin Inhibits Cell Migration Following Previous HMGB1 Exposure

NIH3T3 murine fibroblasts (2×10^4) were seeded on fibronectin-coated inserts placed in 24-well plates. Bottom compartments contained serum-free medium (negative control) or medium with chemoattractant (25 nM HMGB1 or 2.5 nM fMLP as positive control). To examine the effect of GR upon cell migration, the compound was added to both compartments (25, 50 and 100 μM). After 4 h, increased number of migrating cells was observed following fMLP chemoattractant (Fig. 3). Similar increase was noted following HMGB1 addition. Treatment with GR (50 or 100 μM) diminished the numbers of migrating cells to levels seen in cultures where no chemoattractants were added. Such effect was not observed following addition of control fMLP chemoattractant, which pointing to specific

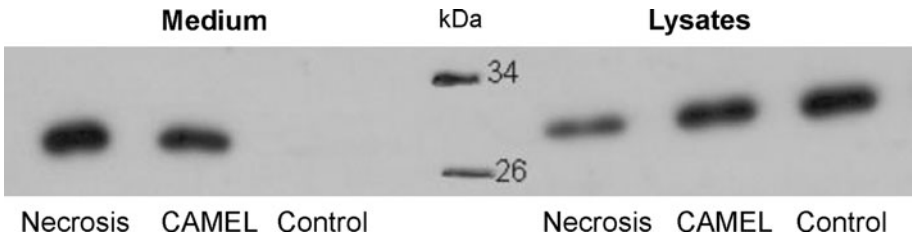

Fig. 1 Identification of HMGB1 in cell lysates and medium from B16-F10 cultures following necrotic type of death. B16-F10 cell cultures were treated with CAMEL (10 μM). After 3 h medium was collected and concentrated whereas cells were lysed. HMGB1 was determined in both medium and lysates: samples were electrophoresed and using Western blotting the released HMGB1 was identified with anti-HMGB1 antibody. Untreated cells served as negative control. Untreated cells subjected to triple freeze/thaw procedure resulting in necrosis (and HMGB1 release) served as positive control for CAMEL-treated cells

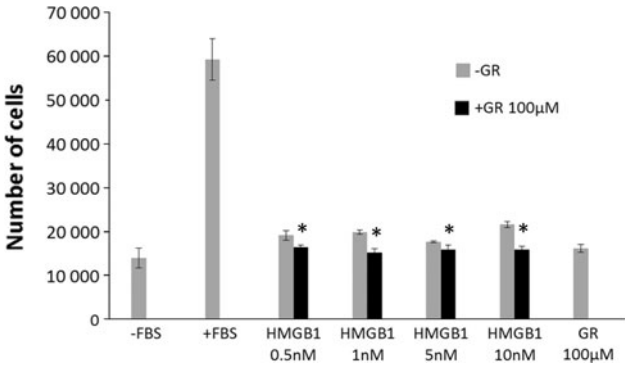

Fig. 2 Effect of HMGB1 cytokine on proliferation of BAEC. BAEC (3×10^3/well) were cultured using 96-well plates with medium containing HMGB1 (0.5, 1 and 5 nM) and GR (100 μM). After 24 h cells were counted. HMGB1 increases cell proliferation throughout the tested concentration range of HMGB1. Addition of GR inhibits this proliferation. Results are mean of three experiments. Differences between HMGB1 and HMGB1 + GR groups are statistically significant (*$p < 0.05$)

effect of inhibitor with respect to HMGB1. Differences in the numbers of migrating cells between HMGB1-treated groups and HMGB1 + GR-treated groups were statistically significant ($p < 0.05$) at 50 and 100 μM inhibitor concentration.

GR Inhibits Proangiogenic Properties of HMGB1

Aortic rings embedded in solidifying fibrinogen-containing agarose were placed in the wells of a four-well plate containing serum-free MCDB 131 culture medium. Cultures were maintained for 1 week at 37 °C/5 % CO_2/95 % air. The ability of aortic rings to form microvessels was tested in the presence of daily-added: epithelial growth factor (EGF) (positive control) and HMGB1 and its inhibitor GR. The number of microvessels formed was highest in the EGF-receiving group (ca. 60) and in the HMGB1-receiving group (ca. 75), respectively (Fig. 4). Addition of GR caused inhibition of microvessel formation to the level seen in control group (no agents added). GR itself had no effect on microvessel formation in aortic rings. Differences in the numbers of microvessels formed in aortic rings between HMGB1-treated groups and HMGB1 + GR-treated groups were statistically significant ($p < 0.05$).

GR Inhibits Inflammatory State Induced by CAMEL

Mice harboring B16-F10 melanoma tumors were injected with necrosis-inducing CAMEL peptide. Selected groups of mice also were administered GR—a HMGB1 inhibitor. Serum levels of TNF-α, an indicator of inflammatory state, were determined. Administration of CAMEL peptide considerably increases serum TNF-α, from 20 pg/mL do 100 pg/mL after 72 h following peptide administration). Administration of GR itself or GR following previous CAMEL injection lowered TNF-α levels to that measured in control mice (Fig. 5). Differences in the amount of TNF-α between the group treated with CAMEL and the group treated with CAMEL + GR was statistically significant ($p < 0.05$).

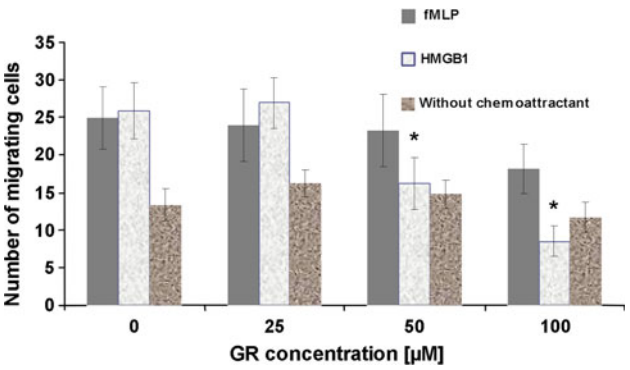

Fig. 3 Effect of HMGB1 and GR on cell migration. NIH cells were suspended in medium without serum and seeded (2×10^4) in Boyden cell. Bottom compartments contained medium either without or with chemoattractant (25 nM HMGB1 or 2.5 nM fMLP/positive control). To check the effect of GR on cell migration various amounts of GR were added to upper compartments (25, 50, 100 μM). After 4 h cells were fixed and Giemsa stained. Migrating cell were counted in five different fields and counts were averaged. Results are mean of three experiments. Differences between HMGB1 and HMGB1 + GR groups were statistically significant (*$p < 0.05$) for 50 and 100 μM concentrations of GR

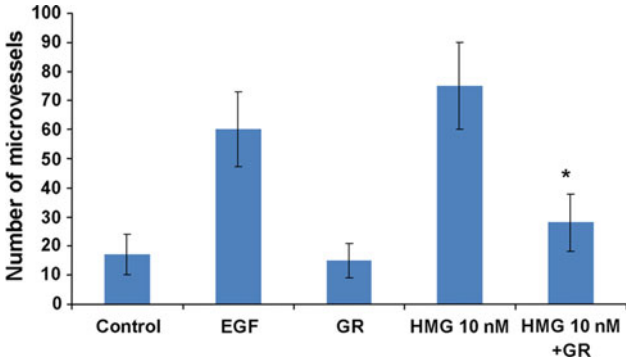

Fig. 4 GR inhibits proangiogenic properties of HMGB1. Aortic rings were placed in fibrinogen solution. Positive control medium contained EGF (10 ng/mL) but no examined agents. Experimental medium contained either HMGB1 (10 nM), or GR (100 μM) or HMGB1 and GR (10 nM and 100 μM, respectively). The agents were administered daily for 1 week. Forming microvessels were monitored. HMGB1 stimulated microvessels' growth. Addition of GR inhibited proangiogenic properties of HMGB1. Differences in the number of microvessels forming from aortic rings between HMGB1 and HMGB1 + GR groups were statistically significant (*$p < 0.05$)

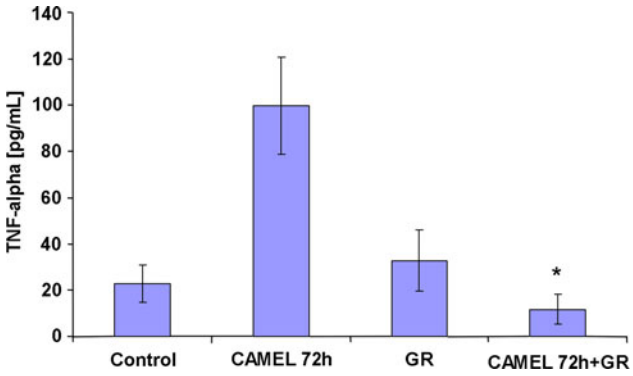

Fig. 5 GR inhibits inflammatory condition triggered by CAMEL-mediated therapy. B16-F10 tumor bearing mice were injected intratumorally with CAMEL, a necrosis-inducing peptide. Selected experimental groups received in addition intraperitoneal injections of GR, an inhibitor of HMGB1 (2 mg/400 μL PBS/animal). After 72 h blood was collected, coagulated and serum isolated and frozen. TNF-α levels (pg/mL serum) were determined in frozen serum samples using TNF-α ELISA kit (BD) according to manufacturer's protocol. Results are mean of five mice in each group. Differences in TNF-α levels between CAMEL and CAMEL + GR groups were statistically significant ($*p < 0.05$)

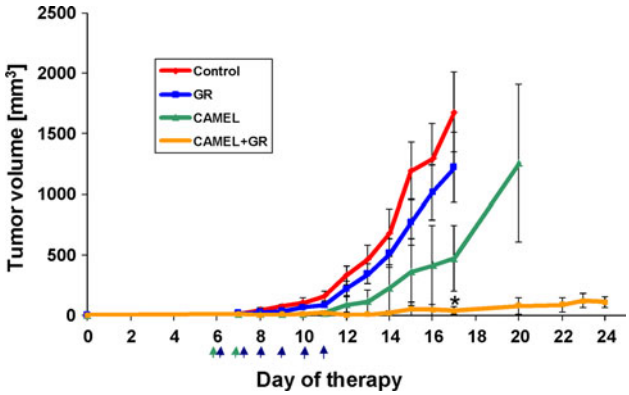

Fig. 6 Anticancer therapy with necrosis-inducing CAMEL peptide and GR an inhibitor of HMGB1 protein. C57BL/6 mice were inoculated with 2×10^5 B16-F10 murine melanoma cells. On day 6 and 7 following inoculation CAMEL peptide (100 μg/mL) was administered intratumorally (*green arrows*) whereas GR (2 mg/animal) was injected intraperitoneally (*blue arrows*). GR (2 mg/animal) was administered for consecutive 4 days. Tumors were measured and growth inhibition was monitored. Combined administration of CAMEL and GR considerably inhibited growth of murine melanoma tumors as compared to monotherapy with either agent or PBS. Results are mean of two experiments; each group numbered five mice. Differences in tumor volumes between CAMEL and CAMEL + GR groups were statistically significant ($*p < 0.05$) on the 17th day of therapy

Combination of Necrosis-Inducing CAMEL and GR, an Inhibitor of HMGB1 Inhibits Growth of B16-F10 Murine Melanoma Tumors

Mice with developed B16-F10 tumors were injected CAMEL (100 μg/mL) intratumorally on day 6 and 7 from inoculation with tumor cells. GR (2 mg/animal) was administered intraperitoneally in experimental group mice. For the following 4 days, GR only (2 mg) was given. Administration of CAMEL peptide only inhibited tumor growth. Administration of GR alone inhibited tumor growth only slightly. On the other hand, differences in tumor growth between the combination of CAMEL + GR versus either agent alone (or PBS) were statistically significant (Fig. 6). Differences in tumor volume between CAMEL-treated group and CAMEL + GR group were statistically significant starting from the 17th day of treatment ($p < 0.05$).

CAMEL and GR, a HMGB1 Inhibitor Decrease Activity of HMGB1, Lower Macrophage Influx and Inhibit Tumor Growth

Mice with developed B16-F10 melanoma tumors (6th day after inoculation with cancer cells) were injected intratumorally with CAMEL (100 μg/mL) and intraperitoneally with GR (2 mg/animal). For the following 4 days, GR only (2 mg) was given. Tumors were then excised and paraffin-fixed. The collected samples were stained with hematoxylin and eosin, an anti-HMGB1 antibody and F4/80 anti-macrophage antibody. H&E staining revealed appearance of extensive necrosis in the wake of CAMEL treatment as well as infiltration of immune system cells (Fig. 7b). Administration of GR alone did not yield marked changes (Fig. 7c), whereas the combination of both agents induced necrosis, but not the infiltration of immune system cells (Fig. 7d). Identification of HMGB1 protein in the preparations shows that during necrosis (following CAMEL administration) this protein is released from cell nucleus into extracellular milieu (Fig. 7f) whereas in the control group HMGB1 remains in cell nucleus (Fig. 7e). Following GR treatment, HMGB1 protein remains in cell nuclei (Fig. 7g). Combination of both drugs markedly decreased the amount of HMGB1 protein present in extracellular space, as compared to the group receiving peptide alone (Fig. 7h). Macrophages present in untreated tissue sections were identified (Fig. 7i). Administration of CAMEL increased the influx of macrophages to necrotic areas (Fig. 7j). GR administration, on the other hand, depleted the number of macrophages present, as compared to that in untreated mice (Fig. 7k). Combining both drugs resulted in a decreased number of macrophages as compared to that in the group receiving CAMEL (Fig. 7l). Photographs of tumor-bearing mice were taken on the 15th day of therapy. The largest tumors were present in control group mice (Fig. 7m). CAMEL administration decreased tumor volume significantly (Fig. 7n). GR alone only slightly inhibited melanoma tumor growth (Fig. 7o). Strongest

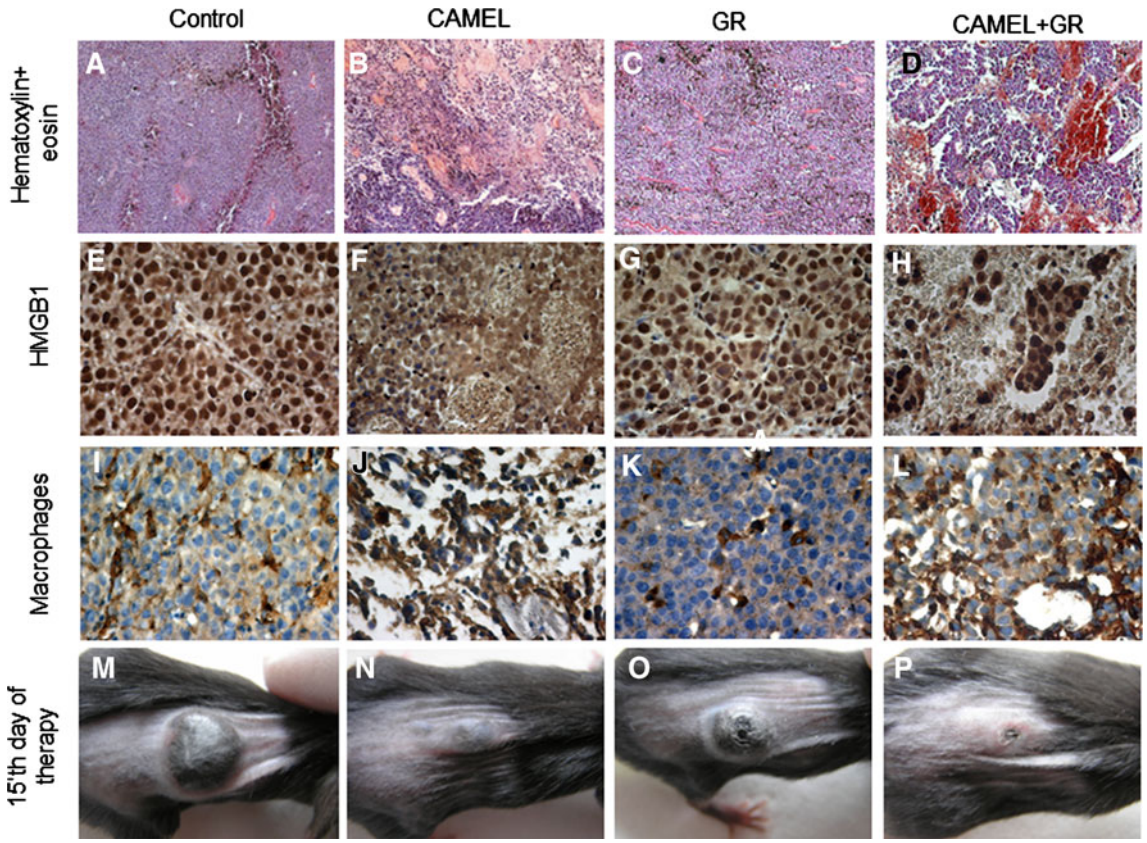

Fig. 7 Immunohistochemical aspects of a anticancer therapy with necrosis-inducing CAMEL peptide and GR, an inhibitor of HMGB1 protein. C57BL/6 mice with developed melanoma tumors (6th day following inoculation) CAMEL peptide (100 μg/mL) was administered intratumorally whereas GR (2 mg/animal) was injected intraperitoneally. GR (2 mg/animal) was administered for consecutive 4 days. Tumors were resected and paraffin-embedded. Next, 5 μm-thick tumor sections were stained with hematoxylin and eosin, an anti-HMGB1 antibody as well as an anti-macrophage F4/80 antibody. CAMEL administration induces necrosis (Fig. 7b), release of HMGB1 into extracellular space (Fig. 7f) and macrophage influx (Fig. 7j). Combined treatment with GR inhibits macrophage influx (Fig. 7l), lowers the amount of HMGB1 present in intercellular spaces as compared to peptide alone group (Fig. 7h). The combination of both agents induced necrosis but no influx of immune system cells was seen (Fig. 7d). Photograph of tumor-bearing mice was done on day 15th. Lens magnification ×10 (**a**–**d**); lens magnification ×40 (**e**–**l**)

arrest of tumor growth was observed following combination therapy involving CAMEL and GR (Fig. 7p).

Discussion

It is well known that agents inducing necrotic type of cell death also inhibit growth of tumors. In addition, they cause release of HMGB1 protein, which is responsible for tumor regrowth (remission). In order to arrest this process two therapeutic approaches are possible. First involves reinforcement of the immune system response via administration of a strong inducer of inflammatory condition, lymphocyte influx and macrophages, such as interleukin (IL)-12. We demonstrated the therapeutic effect of this cytokine in our previous study (Smolarczyk et al. 2010). Another approach is to inhibit the inflammatory condition and all related processes via blocking the activity of HMGB1 protein.

Released HMGB1 stimulates surviving cancer cells to divide and quickly restore their pool. HMGB1 plays also a significant role in angiogenesis. It stimulates the growth of tumor blood vessels and reconstruction of the whole tumor vascular network (Tang et al. 2010). HMGB1 is also a chemoattractant mobilizing progenitor endothelial cells contributing to de novo vasculogenesis (Palumbo et al. 2004; Srikrishna and Freeze 2009). Mitola et al. (2006) had previously shown that HMGB1, when released into extracellular space, strongly stimulates processes leading to blood vessel formation, contributing to regeneration of the damaged cancer tissue.

HMGB1 also activates cancer cells to relocate. Cells that had acquired such ability can migrate to distant regions where they can form metastases in appropriate niches

which provide cancer cells with suitable growth conditions (Psaila and Lyden 2009).

An important role in tumor growth is ascribed to inflammatory state. Such condition does not lead, however, to the destruction of cancer cells. Contrarily, it leads to stimulation of tumor growth. Released HMGB1 activates tumor associated macrophages and cancer cells resulting in secretion of proinflammatory cytokines such as TNF-α, interferon (IFN)-γ, and IL-1β. This further stimulates division of cancer cells as well as endothelial cells, the latter leading to formation of new blood vessels (Le Bitoux and Stamenkovic 2008; Srikrishna and Freeze 2009).

Therefore, it appears justified to therapeutically explore inhibitors of HMGB1 activity, such as, GR, a triterpenoid saponin glycoside of glycyrrhizic acid (3-*O*-(2-*O*-β-D-glucopyranuronosyl-α-D-glucopyran-uronosyl)-18β-glycyrrhetinic acid). GR is a natural product found in roots of *Glycyrrhiza glabra* (licorice). According to Mollica et al. (2007), GR can specifically bind HMGB1 and inhibit its activity (Girard 2007).

Administration of GR (100 μM) completely blocks HMGB1-induced proliferation of BAEC. Similar effect had been observed in case of vessel-associated stem cells (mesoangioblasts) treated with 25–200 μM GR (Mollica et al. 2007). Also, box A domain of HMGB1 shows effect analogous to that of GR, i.e., it inhibits cell proliferation stimulated by HMGB1 (Palumbo et al. 2004). Therefore, blocking the activity of HMGB1 leads to therapeutic effect through inhibition of proliferation of endothelial cells and limiting tumor growth. Besides this, there was not any stimulatory effect on NIH3T3 murine fibroblasts, Heca10 murine endothelial cells and a negligible effect on bEND.3 endothelial cells (data not shown). Van Beijnum et al. (2012) showed that HMGB1 had no effect on proliferation of endothelial cells, but that it promoted endothelial cell migration and sprouting. This suggests that the effect of released HMGB1 is mainly in stimulating migration and inflammatory response which, in consequence, stimulate angiogenesis.

GR also inhibits migration of cells stimulated by HMGB1. Administration of the former completely abrogates chemotactic effect of HMGB1 on migrating fibroblasts. Their migratory behavior, as well as that of mesoangioblasts, was shown to be inhibited by administration of GR or box A of HMGB1 protein (Mollica et al. 2007; Palumbo et al. 2004). GR acts also in antiangiogenic manner; it blocks formation of novel blood vessels from previously existing ones. Appearance of novel microvessels in rat aortic rings was reduced to that of control not receiving any stimulants of this process. Antiproliferative and antimigratory (endothelial stem cells) properties of GR cause angiogenetic processes that follow tissue damage, and limit release of HMGB1 (Mitola et al. 2006; Palumbo et al. 2004).

GR has been known as a strong anti-inflammatory agent (Lamore et al. 2010; Mollica et al. 2007). It is used in heavy inflammation conditions, such as sepsis (Zhu et al. 2010). We show that administration of GR decreased the amount of TNF-α present in serum, the latter being an inflammatory condition marker. Inhibition of inflammation induced by released HMGB1 constrains regenerative properties of this protein affecting angiogenesis and growth stimulation mediated by released proinflammatory cytokines such as TNF-α, IL-1β, IFNγ or IL-6 (Coffelt and Scandurro 2008; Srikrishna and Freeze 2009).

Intraperitoneal administration of GR (2–5 mg/mouse) is shown to inhibit growth of experimental B16-F10 melanoma tumors. The arrest is not statistically significant (data not shown). Mice treated with CAMEL alone showed significant tumor growth after peptide therapy. The same effect was observed in our earlier paper with CAMEL peptide (Smolarczyk et al. 2009). However, combining the HMGB1 inhibitor with necrosis-inducer such as CAMEL yielded considerable, statistically significant inhibition of tumor growth, resulting in complete tumor eradication in some cases. This happens probably because GR inhibits angiogenesis and tumor regrowth which is a consequence of inhibiting cell motility and immunological response. There is also a negligible effect of GR-stimulated tumor growth inhibition. This is probably due to inhibition of HMGB1 found normally in tumor necrotic areas.

Administration of CAMEL in B16-F10 tumor-bearing mice triggers HMGB1 release from cancer cells. Application of GR inhibits activity of HMGB1 and leads to its considerable depletion in tumor tissue. In addition, CAMEL stimulates influx of immune system cells, especially that of macrophages. These, in turn, are capable of stimulating active release of HMGB1, leading to regeneration of therapeutically damaged cancer tissue (Raucci et al. 2007). The role of GR is to substantially inhibit such influx and prevent tumor regrowth.

Anticancer peptides seem to be useful therapeutics because of easy synthesis, low immunogenicity, deep penetration of target tissue and possible specificity (Smolarczyk et al. 2009). However, most of them induce necrotic type of cell death and thereby lead to release of HMGB1 protein, followed by quick tissue regeneration and tumor regrowth. Interference with these processes by GR, an inhibitor of HMGB1, considerably inhibits tumor regrowth after termination of therapy (Fig. 8). In

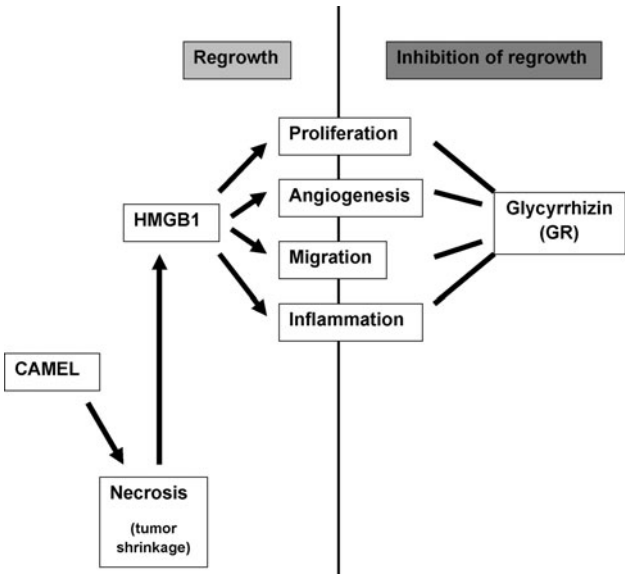

Fig. 8 Inhibition of HMGB1 activity by GR also affects inhibition of tumor growth. Administration of necrosis-inducing CAMEL peptide results in considerable tumor shrinkage. However, HMGB1 protein released during necrosis stimulates tumor regrowth by activating proliferation, angiogenesis, migratory behavior and inflammatory state. Inhibition of HMGB1 activity by GR inhibits, in turn, tumor regrowth

conclusion, combining a necrosis inducer with an HMGB1 inhibitor is shown to be a promising avenue in developing anticancer therapeutic strategies.

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References

Campana L, Bosurgi L, Rovere-Querini P (2008) HMGB1: a two-headed signal regulating tumor progression and immunity. Curr Opin Immunol 20:518–523

Coffelt SB, Scandurro AB (2008) Tumors sound the alarmin(s). Cancer Res 68:6482–6485

Ellerman JE, Brown CK, de Vera M et al (2007) Masquerader: high mobility group box-1 and cancer. Clin Cancer Res 13:2836–2848

Gauley J, Pisetsky DS (2009) The translocation of HMGB1 during cell activation and cell death. Autoimmunity 42:299–301

Girard JP (2007) A direct inhibitor of HMGB1 cytokine. Chem Biol 14:345–347

Hanahan D, Weinberg RA (2011) The hallmarks of cancer: the next generation. Cell 144:646–674

Klune JR, Dhupar R, Cardinal J et al (2008) HMGB1: endogenous danger signaling. Mol Med 14:476–484

Lamore SD, Cabello CM, Wondrak GT (2010) HMGB1-directed drug discovery targeting cutaneous inflammatory dysregulation. Curr Drug Metab 11:250–265

Le Bitoux MA, Stamenkovic I (2008) Tumor-host interactions: the role of inflammation. Histochem Cell Biol 130:1079–1090

Lotze MT, Tracey KJ (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 5:331–342

Mitola S, Belleri M, Urbinati C et al (2006) Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine. J Immunol 176:12–15

Mollica L, De Marchis F, Spitaleri A et al (2007) GR binds to high-mobility group box 1 protein and inhibits its cytokine activities. Chem Biol 14:431–441

Nicosia RF, Ottinetti A (1990) Growth of microvessels in serum-free matrix culture of rat aorta. Lab Invest 63:115–122

Palumbo R, Sampaolesi M, De Marchis F et al (2004) Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J Cell Biol 164:441–449

Psaila B, Lyden D (2009) The metastatic niche: adapting the foreign soil. Nat Rev Cancer 9:285–293

Raucci A, Palumbo R, Bianchi ME (2007) HMGB1: a signal of necrosis. Autoimmunity 40:285–289

Rovere-Querini P, Capobianco A, Scaffidi P et al (2004) HMGB1 is an endogenous immune adjuvant released by necrotic cells. EMBO Rep 5:825–830

Scaffidi P, Misteli T, Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418:191–195

Schlueter C, Weber H, Meyer B et al (2005) Angiogenetic signaling through hypoxia: HMGB1: an angiogenetic switch molecule. Am J Pathol 166:1259–1263

Sims GP, Rowe DC, Rietdijk ST et al (2010) HMGB1 and RAGE in inflammation and cancer. Annu Rev Immunol 28:367–388

Smolarczyk R, Cichoń T, Szala S (2009) Peptides: a new class of anticancer drugs. Postepy Hig Med Dosw 63:360–368

Smolarczyk R, Cichoń T, Kamysz W et al (2010) Anticancer effects of CAMEL peptide. Lab Invest 90:940–952

Srikrishna G, Freeze HH (2009) Endogenous damage-associated molecular pattern molecules at the crossroads of inflammation and cancer. Neoplasia 11:615–628

Tang D, Kang R, Zeh HJ 3rd et al (2010) High-mobility group box 1 and cancer. Biochim Biophys Acta 1799:131–140

van Beijnum JR, Nowak-Sliwinska P, van den Boezem E et al (2012) Tumor angiogenesis is enforced by autocrine regulation of high-mobility group box 1. Oncogene. doi:10.1038/onc.2012.49

Yang H, Tracey KJ (2010) Targeting HMGB1 in inflammation. Biochim Biophys Acta 1799:149–156

Yang H, Wang H, Czura CJ et al (2005) The cytokine activity of HMGB1. J Leukoc Biol 78:1–8

Zhu S, Li W, Ward MF et al (2010) High mobility group box 1 protein as a potential drug target for infection- and injury-elicited inflammation. Inflamm Allergy Drug Targets 9:60–72