**Human α-Lactalbumin Made Lethal to Tumor Cells (HAMLET) Kills Human Glioblastoma Cells in Brain Xenografts by an Apoptosis-Like Mechanism and Prolongs Survival**

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**ABSTRACT**

Malignant brain tumors present a major therapeutic challenge because no selective or efficient treatment is available. Here, we demonstrate that intratumoral administration of human α-lactalbumin made lethal to tumor cells (HAMLET) prolongs survival in a human glioblastoma (GBM) xenograft model, by selective induction of tumor cell apoptosis. HAMLET is a protein-lipid complex that is formed from α-lactalbumin when the protein changes its tertiary conformation and binds oleic acid as a cofactor. HAMLET induces apoptosis in a wide range of tumor cells in vitro, but the therapeutic effect in vivo has not been examined. In this study, invasively growing human GBM tumors were established in nude rats (Han:nrrnu/nrrnu Rowett, n = 20) by transplantation of human GBM biopsy spheroids. After 7 days, HAMLET was administered by intracerebral convection-enhanced delivery for 24 h into the tumor area; and α-lactalbumin, the native, folded variant of the same protein, was used as a control. HAMLET reduced the intracranial tumor volume and delayed the onset of pressure symptoms in the tumor-bearing rats. After 8 weeks, all α-lactalbumin-treated rats had developed pressure symptoms, but the HAMLET-treated rats remained asymptomatic. Magnetic resonance imaging scans revealed large differences in tumor volume (456 versus 63 mm³). HAMLET caused apoptosis in vivo in the tumor but not in adjacent intact brain tissue or in nontransformed human astrocytes, and no toxic side effects were observed. The results identify HAMLET as a novel candidate in cancer therapy and suggest that HAMLET should be additionally explored as a novel approach to controlling GBM progression.

**INTRODUCTION**

Most intracranial neoplasms originate from neuralglial cells and form the heterogeneous group known as gliomas (1). They account for more than 60% of all primary brain tumors and have an unfavorable prognosis (2–4). Glioblastomas (GBMs) are the most malignant of the gliomas with a mean survival time of less than 1 year (4), and they constitute approximately one-fourth of all intracranial tumors.

In recent years, the surgical treatment of brain tumors has made significant technical advances. Microsurgery, neuro-navigation, and new high resolution imaging techniques have reduced surgical morbidity, but the survival time has not improved. The GBMs remain inaccessible to complete surgical removal due to their invasive nature and diffuse infiltrating growth. As a consequence, the current treatment of these patients is palliative, involving partial tumor resection, radiotherapy, and chemotherapy (5).

**MATERIALS AND METHODS**

**Preparation of HAMLET.** HAMLET was produced from apo α-lactalbumin by ion exchange chromatography on a DEAE-Trisacryl M (BioSepra, Cergy-Saint-Christophe, France) column preconditioned with the C181:9 cis fatty acid (13). 125I-labeling of HAMLET (1 mg/ml) was by the lactoperoxidase method (12). For real-time confocal microscopy, HAMLET was conjugated to Alexa Fluor 568 (Molecular Probes Inc., Eugene, OR).

**Cellular Interactions.** The cell lines were cultured as described previously (12), detached, harvested, washed, and exposed to HAMLET or α-lactalbumin. Cell viability was determined by trypan blue exclusion (percentage of dead cells/100 counted cells). Glioma cell line D54 was a gift from Darrel D-Bigner (Duke University, Durham, NC). U251 and CRL2365 were obtained from American Type Culture Collection. A single cell suspension of differentiated murine brain cells was prepared by placing tissue in DMEM (Life Technologies, Inc. Ltd., Paisley, Scotland, United Kingdom) with 1% trypsin and 0.25% DNase in 1% FCS for 30 min at room temperature. After repeated washing, the cells were suspended in DMEM at 4 × 10⁶/ml. The viability was >99%. Confocal microscopy was in an MRC-1024 confocal system attached to a Eclipse 800 upright microscope (Nikon, Kanagawa, Japan).

**Nontransformed human astrocytes CC-2565 (Cambrex, La Jolla, CA) were cultured according to the manufacturer’s instructions. After harvesting from tissue culture flasks, cells were allowed to attach to 8-well glass slides (Nalge Nunc, Naperville, IL) or 24-well cell culture plates for 2 h in astrocyte basal medium (Cambrex) supplemented with ascorbic acid, recombinant human epidermal growth factor (rhEGF), GA-1000, insulin, t-glutamin, and 3% FBS. The adherent cells were washed once with PBS and then exposed to 0.03 mM of Alexa Fluor labeled HAMLET in RPMI 1640 without FCS. Confocal microscopy was in an LSM 510 META confocal system (Carl Zeiss, Germany).**

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Fig. 1. HAMLET structure and function in vitro. A, HAMLET is formed from native \(\alpha\)-lactalbumin by removal of Ca\(^{2+}\) and by addition of the C18:1, 9 cis fatty acid (13). The figure is based on the \(\alpha\)-lactalbumin crystal structure (33). B, sensitivity of tumor cell lines to HAMLET. LD\(_{50}\), concentration required to kill 50% of the cells in 6/24 h. C, dose response of glioma cell lines to HAMLET. Murine primary brain cell cultures were used as controls. D, resistance of nontransformed human astrocytes to HAMLET treatment (0.03 mM). E, cellular trafficking of HAMLET in malignant glioma cells. HAMLET (red, top panels) binds to the tumor cell surface, invades their cytoplasm, translocates to the perinuclear region, and accumulates in the nuclei. Morphological changes include membrane blebbing, nuclear condensation, vesicle formation, cell shrinkage, and formation of apoptotic bodies (light transmission; bottom panels, magnification, \(\times180\)). \(\alpha\)-Lactalbumin binds to the surface and enters the cytoplasm in small amounts but does not relocate to the nucleus. Trypan blue marks dead cells. F, localization of HAMLET in nontransformed human astrocytes. HAMLET enters the cytoplasm, forming small aggregates, but does not relocate to the cytoplasm. The cells remain viable for at least 24 h.
Jena, Germany), and cell viability was determined by trypan blue exclusion and morphological criteria.

Tumor Tissues. Tumor biopsies were collected, with the approval of the Medical Ethics Committee at the Haukeland University Hospital (Bergen, Norway), from a GBM of the right frontal lobe and a para-sagittal meningioma. Biopsy spheroids with a diameter of 300 μm were cultured and used for transplantation (15).

Xenotransplantation of Human GBMs to Nude Rats. All experiments were approved by The National Animal Research Authority and conducted according to The European Convention for the Protection of Vertebrates Used for Scientific Purposes. Nude rats (Han:nu/nu Rowett) bred at the Haukeland Hospital (Bergen, Norway) were anesthetized by i.p. injection of Equitivan and placed in a stereo-tactic frame (model 900; David Kopf, Tujunga, CA) for trepanation, and about 5–10 μl of PBS containing five biopsy spheroids were injected into the striatum. The rats were monitored daily until they developed symptoms of increased intracranial pressure such as passivity, clumsiness, and paresis. The tumor mass was quantified by magnetic resonance scans using a 1.5-Tesla Magnetom Vision instrument (Siemens, Erlangen, Germany) and with a finger-coil for cerebral analysis. The mean time from transplantation of about 0.5 million cells to pressure symptoms was about 2 months, at which time the animals were sacrificed.

CED of HAMLET to the Intact Brain. HAMLET or α-lactalbumin (0.7 mM in 0.15 M NaCl) was administered through a 26-gauge cannula connected to an osmotic mini pump (AD101; Alzet Inc., Mountainview, CA). The region of the tumor was infused at 8 μl/h over 24 h before the cannula was removed. 125I radio-labeled HAMLET (0.7 mM in 0.15 M NaCl, 2–10 × 106 parts/million) was administered as described (18). The distribution of HAMLET was verified by autoradiography on serial brain sections from the entire brain.

Tissue Analysis. Brains were rapidly embedded in Tissue-Tec (Sakura Finetek Inc., Torrance, CA) and frozen in liquid nitrogen. Serial axial 10-μm sections were cut on a Reichert Jung Cryostat (Reichert, Vienna, Austria). Apoptotic cells were detected by the terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) assay (Roche, Basel, Switzerland) and cover-slipped with a mounting medium (Vectorshield; Vector Labs Inc., Burlingame, CA). Cell nuclei were counterstained with propidium iodide (10 μg/ml, for 30 s) and examined in a Leica scanner. Parallel sections were stained with H&E and mounted in Entellan (Merck, Darmstadt, Germany). Sections without freezing artifacts and with an acceptable signal:noise ratio for FITC (TUNEL) and tetramethylrhodamine isothiocyanate (propidium iodide) were identified, and one representative section from the center of each tumor or spheroid was subjected to morphometric analysis. FITC- and tetramethylrhodamine isothiocyanate-positive nuclei profiles were clearly visible above background and were counted from printed pictures. Results are expressed as TUNEL positive in percentage of propidium iodide-positive nuclei.

In Vitro Treatment of GBM Spheroids. Established spheroids (four to five in each group) were moved to serum-free medium, incubated for 3 h with HAMLET or α-lactalbumin, and immediately transplanted into the brains of nude rats. For analysis of apoptosis, spheroids were transferred back to DMEM, incubated for another 21 h, and examined after serial sectioning by the TUNEL assay with morphometry.

Toxicity Tests. Rats receiving HAMLET (0.7 mM), α-lactalbumin (0.7 mM), or NaCl (0.15 M) were analyzed 3 weeks post infusion. The brain was analyzed by magnetic resonance imaging (MRI) scans, using a 1.5-Tesla Siemens Magnetom Vision instrument and with a finger-coil for cerebral analysis. Histopathology was determined as described above using H&E. Biochemical markers of liver and kidney function and C-reactive protein were quantified. The body weight was recorded before infusion and 3 weeks post infusion. Brain function was assessed by the open-field test. Rats were placed in an open-field box (100 × 100 cm) surrounded by black walls (20 cm). The floor was divided into 25 identical sectors (20 × 20 cm) by white stripes. The animals were placed in the central sector, and their movements were scored manually for 6 min. Each motility count represented the crossing of a sector border with both hind limbs, and the direction was noted as right or left. The experiments were performed between 10 a.m. and 2 p.m. in a soundproof room, in a blinded manner.

Statistical Analysis. Groups were compared with t test, one-way ANOVA (post hoc LSD), and survival was described by Kaplan-Meier analysis.

RESULTS

HAMLET Kills Malignant Glioma Cells. The HAMLET sensitivity of glioma tumor cells was first studied in cell culture. Three different glioma cell lines (D54, U251, and CRL2365) were compared with nontransformed murine and human astrocytes. The glioma cell lines died in response to HAMLET at 0.03 mM (Fig. 1, B and C). The nontransformed murine brain cells remained viable also at concentrations of >0.35 mM (Fig. 1, B and C). Furthermore, nontransformed human astrocytes were subjected to HAMLET (Fig. 1D). No apoptotic response was observed, and the cells remained viable for at least 24 h after HAMLET exposure.

By real-time confocal microscopy, HAMLET was shown to invade the tumor cells, forming large cytoplasmic aggregates that moved to the nuclei (Fig. 1E). The nuclei became condensed, and there was cytoplasmic blebbing and formation of cellular fragments resembling apoptotic bodies. In the native conformation, α-lactalbumin bound weakly to the tumor cell surface, and very small amounts of the protein reached the cytoplasm (Fig. 1E), but α-lactalbumin did not form aggregates or move to the nuclei. The human astrocytes were able to take HAMLET into the cytoplasm, where smaller aggregates were formed, but there was no additional translocation of HAMLET to the nuclei (Fig. 1F).

HAMLET Induces Apoptosis in GBM Biopsy Spheroids in Vitro. The ability of HAMLET to induce apoptosis in GBM tissue was verified in vitro. Biopsy spheroids were exposed to HAMLET, and apoptotic cells were identified by the TUNEL assay, with propidium iodide counterstaining to visualize the total cell population. After HAMLET treatment, the GBM spheroids showed abundant TUNEL staining (Fig. 2A). By morphometry, 93% ± 7% (mean ± SD) of the nuclei were found to be apoptotic. TUNEL-positive cells were observed throughout the GBM spheroids at concentrations of ≥0.35 mM. By histopathology, pyknotic and condensed nuclei were observed in the HAMLET-exposed GBM spheroids (Fig. 2A, arrow).

GBM spheroids from the same tumor were treated with α-lactalbumin. A few apoptotic cells were shed from the surface, but no TUNEL-positive cells were seen in the interior of the spheroids, and there was no difference in the frequency of apoptotic cells between the GBM spheroids exposed to α-lactalbumin and the medium control. Both were significantly lower than the HAMLET-treated spheroids (P < 0.0001).

Spheroids derived from a benign meningioma were subjected to HAMLET treatment under the conditions described above. These spheroids were included as a control to further understand the apparent selectivity of HAMLET for the malignant tumor cells. No increase in apoptosis was observed in the meningioma spheroids, as compared with the medium control (Fig. 2B).

HAMLET Inhibits the Growth of Human Glioma Xenografts. The xenograft model is relevant to human GBM disease and may be combined with convection-enhanced delivery (CED) of therapeutic molecules into the tumor area (16–19). In this study, experimental GBMs were established by xenotransplantation of human GBM biopsy spheroids into the nude rat brain (15, 16). The xenografts showed the infiltrative growth characteristics of human GBM, and the control rats developed symptoms after about 2 months (Fig. 3).

CED was used to administer HAMLET (0.7 mM) into the xenografted area of the brain. Native, folded α-lactalbumin served as a control. Before treatment, the tumor cells were allowed 1 week to become integrated into the host brain. HAMLET or α-lactalbumin were then administered by CED for 24 h (Fig. 3A). Two animals in each group died during anesthesia, and four animals in each group...
HAMLET-treated GBM cells were TUNEL positive compared with obtained 12 h after completion of CED showed that 33% which labels DNA strand breaks. Morphometric analysis on tissues in vivo Apoptosis induction was examined pallisading. GBM tumors, showing polymorphic cell morphology and pseudo-rats eventually developed pressure symptoms and died with typical /E, P/ remained asymptomatic (Fig. 3 sacrificial. At this time, all animals in the HAMLET-treated group developed symptoms on day 59, and by day 65, all animals had been sacrificed. At this time, all animals in the HAMLET-treated group remained asymptomatic (Fig. 3E, P < 0.001). The HAMLET-treated rats eventually developed pressure symptoms and died with typical GBM tumors, showing polymorphic cell morphology and pseudo-pallidasing.

Selective Tumor Cell Apoptosis in Human GBM Xenografts. Apoptosis induction was examined in vivo using the TUNEL assay, which labels DNA strand breaks. Morphometric analysis on tissues obtained 12 h after completion of CED showed that 33% ± 7% of the HAMLET-treated GBM cells were TUNEL positive compared with 2% ± 2% in the α-lactalbumin group (Fig. 4, P < 0.001). The apoptotic effect was confirmed by histopathology, which showed typical pyknotic and condensed nuclei in the HAMLET-treated animals (Fig. 4). The host brain surrounding the tumor showed no evidence of apoptosis or necrosis after CED of HAMLET or α-lactalbumin (Fig. 4).

In Vitro Pretreatment of GBM Spheroids Confirmed the Therapeutic Effect. GBM biopsy spheroids were exposed to HAMLET or α-lactalbumin in vitro for 3 h, xenotransplanted, and the brains were examined by MRI scans after 2 months (data not shown). Tumors developed in all control rats that received α-lactalbumin-treated spheroids. The rats developed symptoms on day 59, and after 8 weeks, the mean tumor size was 496 (range 286–696) mm³. At this time, four of the rats with the HAMLET-treated spheroids had no detectable tumors. Two rats had detectable tumors, but they were smaller than in the α-lactalbumin controls with a mean volume of 31 (range 28–34) mm³, and those rats developed pressure symptoms after 84 days. The remaining animals were tumor free and asymptomatic at the time of sacrifice, 210 days after transplantation (P < 0.01).

HAMLET Reaches the Entire Infused Hemisphere. 125I-radio-labeled HAMLET (2–10 × 10⁶ parts/million) was infused by CED with the needle inserted in the striatum, and the distribution of HAMLET throughout the brain was detected by autoradiography on serial brain sections (Fig. 5). HAMLET was shown to reach the entire infused hemisphere from the forebrain to the mesencephalon, 12 h after completion of the CED.

Therapeutic Concentrations of HAMLET Are Not Toxic for Intact Brain Tissue. Potential brain toxicity of HAMLET was examined by MRI and histopathology 3 weeks after CED into the striatum of healthy rats. α-Lactalbumin or saline served as controls. By MRI, small cystic lesions were seen at the infusion site, but there were no signs of edema or tissue damage in the surrounding brain, including the cortex, which had been penetrated by the infusion cannula (see T₂-weighted scans in Fig. 6A). There were no radiological differences between the HAMLET and the control groups.

By histopathology, the infused brains showed some tissue destruction adjacent to the infusion site, with increased cellularity comprising reactive microglia, macrophages, and a few reactive astrocytes. There were no significant signs of toxicity in the surrounding brain parenchyma and no differences between the HAMLET-treated and the control groups (Fig. 6B).
Biochemical markers and body weight changes were monitored 3 weeks after infusion. No differences were observed among the HAMLET, α-lactalbumin, and NaCl-treated rats (P > 0.05 in all groups; Fig. 6, C and D).

Changes in movement and behavior were assessed by the open-field test 3 weeks after infusion. The rats were placed in an open-field checkerboard, and the number of crossings to a new square was recorded. No significant movement disorders were detected (Fig. 6E).
HAMLET TREATMENT OF HUMAN GLOBLASTOMAS

Fig. 5. Distribution of radiolabeled HAMLET (2—10 × 10^6 parts/million) after infusion into brains of intact rats (n = 3; magnification, ×90). The letters indicate the position of the sections and x the infusion site. a, frontal lobe; b, basal ganglia; c, thalamus; g, substantia nigra.

DISCUSSION

HAMLET possesses two interesting properties that add to its potential as a novel antitumor agent. It kills malignant cells in vitro, but leaves fully differentiated cells unaffected, and it activates programmed cell death rather than necrosis (12). Here, we show that HAMLET maintains these properties also in vivo, killing malignant tumor cells in the brain, but sparing intact brain tissue. In addition, the infusion of HAMLET into established human GBM tumors had a therapeutic effect, since it delayed tumor development and the onset of pressure symptoms.

Two main experimental models have been developed to study GBM treatment in vivo. Although glioma cell lines rapidly form confluent cultures in vitro and invariably produce intracerebral tumors, they are not invasive in vivo, and are thus less suitable as a model of the human disease (20). Biopsy spheroids of human gliomas, in contrast, maintain their invasiveness after xenotransplantation into nude rats (21). The in vitro step is essential to obtain a reproducible tumor mass. The human tumor used in this study caused pressure symptoms after 60 days with little variation.

Infusion of HAMLET into established tumors triggered apoptosis in vivo, as shown by the TUNEL assay and histopathology. There was no evidence of necrosis, and the effect appeared to be selective, with no histopathological changes in the surrounding intact brain. The in vitro studies confirmed the selectivity of HAMLET for malignant cells, and HAMLET was shown to accumulate in the nuclei, suggesting that the interaction with the glioma cells follows the pattern previously observed for other tumor cell types (14). Native α-lactalbumin failed to induce apoptosis in glioma cell lines or spheroids and did not reach the cell nuclei. These direct cellular effects are the most likely explanations for the difference in disease progression between the xenotransplanted rats receiving HAMLET or α-lactalbumin, the same protein but in a different folding state.

Human α-lactalbumin is the most abundant protein in human milk (22), in which it serves as an enzyme specifier in lactose synthesis (23). Removal of Ca^{2+} results in the loss of the native conformation, and HAMLET is produced in vitro by removal of Ca^{2+} and by adding a C18:1 fatty acid, which locks the protein in the active conformation (13). It may be speculated that HAMLET is formed in the stomach of the breast-fed child, in which the low pH of gastric juice promotes unfolding of the protein (24) and triggers pH-sensitive lipases, which release the C18:1 fatty acid from milk phospholipids (25). HAMLET might then act as a natural tumor scavenger in infancy, with the mission of purging atypical or highly immature cells during normal development. Epidemiological studies have shown that breast-fed children have a reduced incidence of e.g., childhood leukemia (26), but we have not found any reports on brain tumor incidence in relation to breast-feeding.

This study provides novel evidence that a protein-folding variant may be used as a therapeutic agent in malignant disease. This may appear paradoxical, because protein folding variants mainly have been recognized as a cause of disease. The disease-causing isoform of prions accumulates in brain tissue, and amyloid is formed when unfolded proteins such as β-amyloid, apolipoprotein, or lysozyme accumulate and disrupt cellular homeostatic functions (27–29). HAMLET, in contrast, appears not to be harmful to normal tissue but to selectively purge malignant and immature cells by apoptosis (14). In this case, a change in α-lactalbumin fold converts this protein from an enzyme specifier in lactose synthesis to an efficient inducer of apoptosis in malignant cells. The results illustrate how changes in tertiary conformation may allow a single DNA sequence to encode a protein with several functions and become involved in the natural defense against cancer.

Malignant GBMs have thus far proven refractory to conventional therapies. As a result of their infiltrating growth pattern, malignant cells are dispersed throughout the brain and are not amenable to selective surgical removal. There is a need for therapeutic agents that selectively eliminate the tumor cells without damaging the surrounding brain. Regional CED of a targeted protein toxin (transferrin-diphtheria) was shown to decrease the tumor volume after 1–14 months (11), but clinical use of transferrin-coupled protein toxins may be limited. Brain capillary endothelial cells are known to express...
significant levels of transferrin receptors potentially causing a cytotoxic effect at the capillary level with concomitant ischemia and an inflammatory response.

The mechanism(s) by which HAMLET induces cell death are not fully understood. In tumor cells, large amounts of HAMLET enter the cytoplasm and move to the nuclei, where HAMLET interacts with histones and disrupts the chromatin (30). Based on this effect, HAMLET may be expected to activate $p53$-dependent rescue mechanism. Because benign brain tumors have intact $p53$ expression whereas the malignant types contain mutated $p53$ (31, 32), the effect of HAMLET on tumor cells would fit with a $p53$-related mechanism. Studies in a variety of different tumor cell lines have shown, however, that the sensitivity to HAMLET is $p53$ independent. Thus, the $p53$ genotype of the GBMs is not likely to explain their sensitivity to HAMLET or the resistance of the benign meningioma cells.

The effects of HAMLET on the established brain tumors are...
promising, because therapeutic concentrations did not harm the normal brain and did not produce any neurological symptoms. Nontransformed human astrocytes were fully resistant to HAMLET, and the complex was not transported to the nuclei in those cells. We conclude that HAMLET has the potential to act as a selective inducer of GBM apoptosis, with regional therapy of HAMLET being a novel approach to control the progression of this highly malignant and invasive central nervous system tumor.

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