

STUDIES ON MODULATED ELECTROHYPERTHERMIA INDUCED TUMOR CELL DEATH IN A COLORECTAL CARCINOMA MODEL

PhD thesis

Nóra Meggyesházi M.D.

Pathological Sciences Doctoral School
Semmelweis University



Supervisor: Dr. Tibor Krenács Ph.D.

Official reviewers:

Dr. Tibor Glasz, Ph.D.

Dr. Gyula Péter Sziget Ph.D.

Head of the Final Examination Committee:

Dr. Péter Sótonyi Member of MTA

Members of the Final Examination Committee:

Dr. Gábor Lotz Ph.D.

Dr. Árpád Csurgay Member of MTA

Budapest, 2015

1. Introduction

1.1. Hyperthermia in oncology

The term hyperthermia refers to techniques of heat application administered to tissues as an adjunct to conventional strategies of cancer treatment such as chemo- or radiotherapy. The aim of hyperthermia treatment is, like any oncological therapies, to completely and selectively destroy the malignant tissue. The main forms of hyperthermia include whole body hyperthermia, hyperthermic perfusion techniques and local/regional hyperthermia. Local hyperthermia is applied to tumors of relatively small size, while regional hyperthermia is used to heat up a body region involving the tumor. For such heating infrared radiation, microwaves, radio waves and ultrasound can be used. Oncological hyperthermia uses heat energy to destroy the malignant cells. The absorbed energy is converted to heat, which then can lead to the increment of temperature. Therefore, one has to distinguish heat (as the absorbed energy) and the resulted elevation of temperature as a consequence of energy absorption. Local/regional hyperthermia works by energy/heat absorption in the targeted tissue volume although *in vivo* blood flow can reduce the efficiency of energy absorption by cooling the heated volume of the tumor tissue.

1.2. Principles of modulated electrohyperthermia

Modulated electrohyperthermia (mEHT) is a non-invasive technique for targeted tumor treatment, using 13.56 MHz radiofrequency with 1/f amplitude modulation.

The bioelectrodynamics properties of biological materials can be simplified to the description of complex permittivity resulted from the dipole content, complex conductivity resulted from the free charges and to the dispersion properties resulted from the distinct relaxation at different frequencies. These electric properties of a given biological material are highly frequency dependent resulting in the selectivity and distinct penetration depth of the electric field at distinct frequencies. Malignant tissues have higher permittivity and conductivity on 13.56 MHz frequency than non-malignant tissues.

The elevated conductivity and permittivity of malignant tissues can be explained by their altered metabolism, a shift towards aerobic glycolysis, also known as the Warburg effect. This leads to increased cellular water and ion content and increased membrane permeability of the cancer cells.

1.3. Characteristics of cell death forms

The “point of no return” in a cell’s fate can usually be characterized by (1) a massive caspase activation, which is part of the classic apoptotic program. However, caspase independent death may also occur, moreover, caspases might also be involved in non-lethal pathways such as cell differentiation and activation. A further feature is the loss of mitochondrial transmembrane potential (2), which is usually preceded by mitochondrial membrane permeabilisation (3) resulting in the liberation of lethal catabolic enzymes or activators. Finally, the phosphatidilserin exposure (4) from the inner to the outer leaflet of the plasma membrane can also be a sign of irreversible cell damage.

Important signs of cell death include the cell loss of plasma membrane integrity (1); blebbing of the cytoplasmic membranes and shrinkage of nuclear chromatin followed by the complete fragmentation of the cell including the nucleus; (2) the occurrence of discrete nuclear bodies (referred as “apoptotic bodies”), finally the engulfment of cells corps by adjacent cells or phagocytes (3) *in vivo*.

There are several subroutines of programmed cell death forms exist including excintric and insintric apoptosis (in a caspase dependent and independent form), necroptosis/regulated necrosis, autophagic cell death, mitotic catastrophe, netosis, parthantos, pyroptosis, anoikis, entosis and cornification.

1.4. Characteristics of immunogenic cell death

Initially, apoptosis was considered as an immunologically silent form of cell death. However, recent evidence has been revealed that some lethal stimuli can lead to cell death through immunogenic effects. Immunogenic cell death (ICD) is a form of programmed tumor cell death through the well-defined spatiotemporal appearance of damage-associated molecular patterns (DAMP), which can trigger anti-tumor immune response. ICD is known to be provoked by massive cell stress in synergy with programmed cell death using chemotherapeutic agents (doxorubicin, oxaliplatin etc.) , cardiac glycosides , hypericin based photodynamic therapy or capsaicin. It is of note, however, that these interventions can generate slightly different DAMP patterns. The DAMP sequence, relevant to inducing ICD in tumor cells include the pre-apoptotic surface exposure of calreticulin, the surface appearance of heat shock proteins (Hsp70 and Hsp 90) and ATP release at early apoptotic stages, followed by passive release of high mobility group box 1 (HMGB1) as well as Hsp70 and Hsp90 release at the late stages.

2. Aims of our studies

- Determining tumor cell damage induced by a single shot of mEHT treatment in an *in vivo* xenograft model set up using HT29, colorectal cancer cell line, in Balb/c (nu/nu) immunocompromised mice.
- Investigating the molecular background of cell death induced by the mEHT treatment in the HT29 xenograft model.
- Characterizing additional, cell stress related molecular changes induced by mEHT in the HT29 xenograft model, which may promote antitumor immune response. Testing for damage associated molecular pattern sequence relevant to immunogenic cell death response.

3. Materials and methods

3.1. Tumor model and treatment conditions

HT29 human colorectal carcinoma cells were xenografted into both femoral regions of Balb/C (nu/nu) mice, followed by a single shot mEHT treatment (13.56 MHz modulated radiofrequency) for 30 minutes. 3 parallel samples were collected at 0, 1, 4, 8, 14, 24, 48, 72, 120, 168, 216 h post-treatment.

3.2. Histomorphological analysis

Whole cross sections from the tumor samples stained for hematoxylin and eosin (H&E) were digitalized using Panoramic Scan (3DHISTECH, Budapest, Hungary) and analyzed with the HistoQuant module of Panoramic Viewer software based on image color and intensity segmentation.

3.3. mRNA chip analysis

Total RNA was isolated from 4h treated and untreated frozen samples together with 24h control samples followed by RNA quality control measurement. The recommendation of Minimum Information About a Microarray Experiment (MIAME) guideline was followed for the microarray analysis. Samples were hybridized on HGU133 Plus2.0 arrays. Feature selections were done according to the log₂FC (log₂ fold change) values to select at least two-fold up/downregulated genes.

3.4. Apoptosis protein array

Proteins were isolated from the 8, 14, 24 h post-treatment frozen samples. The expression of 35 apoptosis-related proteins was tested simultaneously in the treated and untreated samples using a nitrocellulose membrane Proteome Profiler™ Human Apoptosis Array Kit array. Semi-quantitative analysis of signal density was done using ImageJ 1.45s software.

3.5. Immunohistochemistry and immunofluorescence

Formalin-fixed and paraffin-embedded tissue samples were used either for cutting whole sections or for creating tissue microarray (TMA) blocks. Sections were stained using antibodies specific for apoptosis inducing factor (AIF), bax, cleaved caspase-3, cytochrome c, mitochondrial antigen, receptor-interacting serine/threonine-protein kinase (RIP) 1, RIP3, TNF-related apoptosis-inducing ligand receptor (TRAIL-R) 2, Ki-67, calreticulin, HMGB1, Hsp70, Hsp90, CD3, myeloperoxidase (MPO) antibodies the detection was carried out by either horse radish peroxidase conjugated anti-rabbit IgG using 3,3'-diaminobenzidine (DAB) or aminoethylcarbazole (AEC).

or Alexa546 (orange-red) or Alexa488 (green) conjugated anti-rabbit IgG. Nuclei were stained either with hematoxylin for immunohistochemistry or with 4',6 diamidino-2-phenylindole (DAPI) for fluorescent staining.

3.6. TUNEL assay

The DNA fragmentation was measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

3.7. Western blot

Proteins were isolated from frozen tissue samples. Lysates were loaded on polyacrylamide gel and separation was carried out by gel electrophoresis followed by protein blotting on PVDF membrane. The following proteins were measured: caspase-3, caspase-8, RIP1 and AIF.

3.8. Statistical analysis

For analyzing protein expression data related to a single time point that followed normal distribution the independent T-test was used. For time series data analysis the Fridmann test was used followed by the Wilcoxon post-hoc test. The results were significant at $p < 0.05$.

4. Results

4.1. Histomorphological analysis of treatment related tumor cell death

Modulated EHT treatment induced significant tumor destruction in the central zones of the treated HT29 xenografts. The H&E stained cross sections revealed significant tumor destruction between 48-120 h post-treatment with a maximum of 7-fold peak increase at 72 h post-treatment compared to the untreated controls.

4.2. Detection of treatment related DNA fragmentation

Significant treatment related elevation of DNA fragmentation was detected with TUNEL assay 24-48 h post-treatment. In agreement with this, significant nuclear shrinking (pycnosis) and accumulation of dense chromatin fragments (apoptotic bodies) were observed in the treated compared to the untreated tumors between 48-72 h.

4.3. Detection of treatment related differential mRNA expression

An early upregulation (4 h post-treatment) of heat shock proteins (Hsp70 and Hsp90) were observed at mRNA level.

4.4. Detection of treatment related differential protein expression

4.4.1. Apoptosis related protein expression

Mitochondrial accumulation of Bax and mitochondrial to cytoplasmic release of cytochrome c proteins were detected between 8-14 h. The substantial cytoplasmic to nuclear translocation of AIF and its 57 kDa activated fragment detected between 14-24 h post-treatment indicating AIF as an effector for DNA condensation and fragmentation. A secondary upregulation of TRAIL-R2 was observed at 8 and 14 h post-treatment.

Cleaved caspase-3 levels were low and mainly localized to MPO positive myelomonocytic cells primarily of neutrophil granulocytes and CD3 positive immature T lymphocytes.

No significant change was observed in RIP1 or RIP3 protein levels at any time point.

Ki67 immunostaining also revealed no significant difference between treated and untreated tumors concerning the proliferating cell fractions in the morphological intact tumor areas.

4.4.2. Damage associated molecular pattern related protein expression

Modulated EHT treatment related cell death was accompanied by the early membrane translocation of calreticulin protein (4 h post-treatment), followed by the membrane accumulation of Hsp70 between 14-24 h. A transitional decrement at 48 h was observed in

Hsp70 expression with a secondary elevation between 72-120 h post-treatment. Hsp90 appeared in the cytoplasmic membranes only at later time points, at 168 h and 216 h post-treatment. In contrast to Hsp70 kinetics, Hsp90 levels showed continuous elevation after treatment between 24 h and 216 h. HMGB1 release from tumor cell nuclei from 24 h post-treatment and its clearance from tumor cells by 48 h were detected.

4.4.3. Identification of immune cells

The number of MPO positive neutrophil granulocytes (and monocytes) was significantly elevated in the treated samples compared to the untreated controls 48, 72, 120, 168 and 216 h post-treatment. The number of CD3 positive immature T lymphocytes was significantly higher in the treated group at 120 h, 168 h and 216 h post-treatment.

5. Conclusion – New observations

- We have determined the time related tumor destruction in HT29 xenograft model.
- Programmed cell death induced by mEHT in HT29 xenograft dominantly follows a caspase independent subroutine.
- A single shot mEHT treatment related tumor cell stress can generate the spatiotemporal sequence of damage associated molecular pattern (DAMP) signals – including cell membrane exposure of calreticulin, Hsp70 and Hsp90 with nuclear release of HMGB1- in the HT29 colorectal cancer xenograft model without any additional genetic or pharmaceutical intervention.
- Our data suggest that mEHT might be a potential local inducer of immunogenic cell death, without a systemic interference with immune functions.

6. Bibliography of the candidate's publications

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