Oncothermia treatment induced immunogenic cancer cell death

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There are increasing evidence that certain type of anticancer therapeutical methods can induce a very special form of programmed cell death, trigger an immune response against the tumor, so in recent years a new concept of immunogenic cell death (ICD) has emerged [1]. Apoptotic cell death has a vital importance in this process [2], [3], [4]. The immunogenic characteristics of ICD are mainly mediated by damage-associated molecular patterns (DAMPs) [5], [6]. One of the most important key molecules amongs many others are the heat shock proteins (HSPs) [7], [8] and some death receptors [9]. Oncothermia method (OTM) is a long time applied tumor treatment modality in the human clinical practice [10]. Experimental results showed that OTM can effectively and selectively destroy the tumor tissue, even is lower temperatures [11] and the OTM treated tumor cells undergo apoptotic cell death [12]. But recent investigations revealed some unusual immunological aspect of oncothermia treatment taking into a hypothetical consideration that OTM can induce a special form of immunogenic cell death. The theoretical summary of the concept can be seen on Figure 1-3.



Figure 8. The mechanism of ICD. OTM induced programmed cell death and its consequencies



Figure 9. The mechanism of ICD. The process of the DAMP formation, immune-recognition and immune-activation



Figure 10. The mechanism of ICD. The process of the non-specific and specific antitumor response

Materials and Methods

Animal model: HT29 cell line xenografted to both femoral regions of BalbC/nu/nu mice were treated on one side with a single shot OTM treatment (LabEHY, Oncotherm Ltd, Hungary) for 30 minutes of ~1 cm diameter tumors. Sampling was made after 0, 1, 4, 8, 14, 24, 48, 72, 120, 168, 216 h in 3 mice each group by keeping 5 animals as sham treated controls. The experimental animals can be seen on Figure 4.



Figure 11. The HT29 xenograft tumor model mice in the time course experimental design

Sample analysis

1. Histomorphological analysis (HMA) and immunhistochemistry (IHCH) and terminal transferase dUTP Nick End Labeling (TUNEL) assay were performed on whole tumor cross section samples and tissue microarrays (TMA) of triplicate samples. Results were analyzed using digital microscopy (Pannoramic Scan, 3DHISTECH, Budapest).

2. 4h post-treatment mRNA expression was compared to untreated samples by a Total human genome chip (GeneChip human genom U133 Plus 2.0, Affymetrics). The results were analyzed by Bioconductor software.

3. 35 cell death related proteins were screened on 8, 14, 24h treated and on the 24h untreated samples with an apoptosis array chip (R&D Proteome Profiler Human Apoptosis Array), and the results were analyzed using the ImageJ software.

The summary of the tumor sample processing can be seen on Figure 5.



Figure 12. The structure of the tumor sample processing and analysis

Results

1. Oncothermia treatment can induce programmed cell death (apoptosis) in the tumors which create many apoptotic bodies observed by the histomorphological analysis. (see Figure 6.) Tunel assay proved the apoptotic cell death (see Figure 7.). Prsence of apoptotic bodies in a destructed tumor tissue is essential to induce immunogenic reactions by apobody phagocytosis.



Figure 13. Apoptotic body formation 48H post treatment in the OTM treated tumor (standard) HE staining, red circles show the apobodies)



Figure 14. OTM treatment induce programmed cell death (Apoptosis) In the treated tumor sample many TUNEL positive cell nuclei are visable

2. Oncothermia treatment induced cell death is higly immunogenic, showing many aspects of the key molecular pattern dynamic changes what is characteristic of immunogenic tumor cell death. The most important molecular changes are the follows:

A, High Mobility Group Box 1 protein (HMGB1). HMGB1 is the very important hallmark of the ICD. In normal state HMGB1 is located in the cell nuclei, where it stabilizes the nucleus and regulates the transcription of many genes. More and more evidence suggests that it can be released from apoptotic cells. Extracellular HMGB1 act as a cytokine and can can activate DCs (through TLR 4) therefore can trigger anti-tumor T cell responses and mediate ICD. OTM treatment induced programmed cell death accelerate its release to the extracellular matrix (see Figure 8-9.).



Figure 15. Immunohistochemical detection of HMGB1 14h and 24h after the treatment. It is clearly visible the HMGB1 released to the extracellular matrix in OTM treated tumors



Figure 16. Immunofluorescent detection of HMGB1 24h after the treatment

B, Elevated HSP (HSP70 and HSP90) expression rate in the treated tumors after the treatment detected at mRNA level by the gene chip, (see Figure 10.) at protein level by apoptosis array chip and the IHCH (see Figure 11.). There are some histomorphological evidence that the increased level intracellular HSP can be expressed on the cell membrane and can externalize in later time points after the treatment, and can become a strong immunostimulant signal for antigene presenting cells by the process of co- presentation.



Figure 17. OTM treatment induced HSP upregulation at the mRNA level measured by the GeneChip



Figure 18. Immunfluorescent detection of HSP70 72h post-treatment. The HSP70 is expressed on the cell membrane 14H after the treatment and released to the extracellular matrix 72H post treatment

C, Death receptor 5 (TRAIL R2) is a potent antitumor immune reaction inducer and its upregulation detected by apoptosis chip and IHCH, was also found in the treated tumors 14H after the treatment. (see Figure 12.)



Figure 19. Immunofluorescent images about the TRAIL-R2 (DR5)

3. Oncothermia treatment can induce strong and very unusual local immune reaction at the site of the treatment, long time after the electromagnetic intervention. The HMA reveled an appearance of a leukocyte invasion ring around the destructed tumor tissue area. (see Figure 13.).



Figure 20. HE stained whole tumor cross sections 168h post treatment. The red markings indicate the welldefined invasion ring

IHCH proved the presence of MPO + neutrophiles (see Figure 14.) and CD3+ lymphocytes (see Figure 15.) in this peculiar invasion ring. TUNEL assay performed in TMA samples showed high TUNEL positivity in this invasion ring, so we believe, these infiltrating immune cells can induce secondary apoptotic tumor cell death in the intact tumor tissue.



Figure 21. IHCH detection of myeloperoxidase (MPO) from TMA multiblock. MPO is a marker of neutrophyle granulocytes. The leukocyte invasion ring what appears at 72H and became very characteristic at 168H around the destructed tumor area, contains high number MPO positive cells (neutrophils)



Figure 22. IHCH detection of CD3+ positive cells from TMA multiblock. The invasion ring contains a huge amount of CD3+ T cells 168h post treatment

Conclusions

Oncothermia treatment can induce a very special form of programmed cell death, the immunogenic cancer cell death what was proven experimentally. These experimental findings can be the strong scientific theoretical basis to develop a special oncothermia treatment-based immunotherapeutical approach to fight against not just solitaire tumors, but malignant metastatic disease.

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