SYNERGISTIC EFFECTS OF MODULATED ELECTRO-HYPERTHERMIA AND COX-2 INHIBITION IN TRIPLE-NEGATIVE BREAST CANCER AND MELANOMA MOUSE MODELS

PhD thesis

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1. Introduction

Previous results of a multiplex analysis of the mEHT effects at both the gene and protein levels revealed a local acute phase response (lAPR) in 4T1 triple-negative breast cancer (TNBC): the synthesis of acute phase proteins (APP), and the optimization of the innate immune response. Here, we report that mEHT monotherapy in addition to the described reaction stimulates local inflammatory proteins such as interleukin-1 Beta (IL-1β), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2). The lAPR and the IL-1β, IL-6, and COX-2 induction can be considered as part of a self-defensive reaction of tumor cells due to mEHT-induced stress, although the role of the inflammation in cancer is not fully understood. In the present in vivo study, mEHT was combined with nonsteroid anti-inflammatory drugs (NSAIDs; aspirin (ASA) or SC236), which target multiple components of the acute phase reaction, and alone

have already demonstrated promising outcomes in various tumors.

Here, we demonstrated that NSAID treatment synergistically increased the antitumor effect of mEHT in both 4T1 TNBC and B16F10 melanoma mouse models. Besides the inhibition of tumor growth, we have observed an increased tumor destruction ratio (TDR) in combined-treated tumors, which was accompanied by an increase in cleaved caspase-3 (cC3), suggesting that apoptosis plays an important role in the antitumor effect of mEHT and NSAIDs in vivo. To understand the molecular bases of synergism, we performed mRNA studies with RT-PCR and Nanostring. Molecular studies revealed a statistically significant reduction in the IL-1β and COX-2 expression by the combination therapy. Analysis of differentially expressed genes (DEG) using Gene Ontology (GO) through the Database for Annotation Visualization and Integrated Discovery (DAVID).

Our findings suggest that the synergistic combination of mEHT and a selective COX-2 inhibitor may regulate the extracellular matrix turnover and cell membrane protein expression.

Triple-negative breast cancer and melanoma are aggressive, and recurring, with early metastasis compared with most other cancer types. TNBC accounts for 15% of all types of breast cancer. TNBC cells do not express the estrogen receptor, the progesterone receptor, or for the human epidermal growth factor receptor 2; consequently, there are no tumor-specific (such as targeted or hormonal) treatment options, at present. Contemporary treatments for melanoma include surgical resection, chemotherapy, and radiotherapy.

mEHT represents an adjuvant treatment in different cancer models that are recently utilized in clinical medical practice. mEHT contributes to tumor-cell destruction selectively by accumulation of the radiation energy within the tumor without affecting

healthy neighboring tissues. Specific energy accumulation by the tumor cells is partially due to the altered (anaerobic) glucose metabolism of tumor cells (independent of the availability of oxygen called ‘Warburg effect’).

Numerous epidemiological and experimental studies show that NSAIDs reduce the risk, incidence, and mortality in some cancers, including melanoma and breast cancer. Both nonselective (NSAIDs) and selective COX inhibitors (selCOXIBs) have been associated with lower cancer incidence. However, selCOXIBs have demonstrated greater significance than nonselective NSAIDs. NSAIDs inhibit the production of prostaglandins, proinflammatory cytokines, and tumor growth factors. Furthermore, NSAIDs may affect COX-independent inflammatory pathways such as NF-kB, MAPK, mTOR, PDK-1/Akt, and Wnt/b-catenin, by the inhibition of activation of transcription factors. These pathways support cell proliferation and

angiogenesis but suppress apoptosis, thus supporting tumor growth as well as participate in the regulation of the tumor microenvironment (TME). Therefore, COX inhibition influences tumor progression by decreasingmigration, metastasis, angiogenesis, increasing apoptosis, and sensitivity to other conventional anticancer therapies such as chemotherapy, immunotherapy, or radiotherapy. Besides the novel strategy of potential anticancer therapy.

In this hypothesis, it is suggested that NSAIDs may regulate the TME and mEHT-induced proinflammatory cytokines: IL-1β , IL-6, and COX-2. As a result, NSAIDs may enhance the mEHT-induced tumor celldeath. Combining clinically available mEHT with NSAIDs is a new potential tool in oncologic therapy

2. Objectives

Our aims were:

1. To investigate the molecular effects of mEHT in the treatment of 4TI TNBC.
2. Understanding the role of mEHT-induced COX-2, IL-6, and IL-1β expression in 4T1 TNBC.
3. To enhance the anti-tumor effect of mEHT - establish a protocol for 4T1 TNBC and B16F10 melanoma mouse models using combinational treatment of mEHT and COX inhibitors.
4. To gain new, translationally relevant insights that can be used in clinical therapy.

5. Materials and methods

5.1. *In vivo* treatment of 4T1 TNBC

1×10^6 4T1 TNBC cells /50µl PBS were aspirated into Hamilton syringe. Mice were inoculated in the

4th mammary fat pad at the age of six- to eight-weeks. Animals were given a daily dose of 100 mg/kg acetylsalicylic acid or 6 mg/kg selective COX-2 inhibitor (SC236) via intraperitoneal injections. Treatment with COX inhibitors, administered every day during the entire experiment, was combined with mEHT. Mice were treated four times in every 48 hours with a newly constructed labEHY-200 mEHT device. Tumor volume was monitored by ultrasound every day until the termination of the experiment.

5.2. In vivo treatment of B16F10 melanoma

1×10^5 B16F10 melanoma cells were injected into the tail vein of seven- to nine-week-old female C57BL/6 mice that have induced tumor nodules in the lungs. One day after inoculation, mice were treated with mEHT alone or mEHT combined with aspirin at 11.1 mmol/L concentration in their drinking water. Animals have been treated six times using the LabEHY-200 device set up. Animals were

terminated on day 20, 48 hours after the last mEHT treatment. The lung melanoma burden was assessed by counting the number of tumor nodules on the surface of the lungs.

5.3. RNA isolation and real-time PCR

RNA was isolated using TRI reagent according to the manufacturer's protocol. A high-capacity cDNA reverse transcription kit was used to reverse transcribe the isolated RNA. The amplified cDNA was used as template for the RT-PCR. SYBR Green-based qRT-PCR using Sso Advanced ™ Universal SYBR® Green Supermix and the CFX96 Touch Real-Time PCR Detection System was used to detect messenger RNA in the samples. The 18S and GAPDH genes were used as normalising genes.

5.4. Histopathology and immunohistochemistry

Tumor tissues were fixed in 4% formalin and embedded in paraffin (FFPE). Using a polymer-peroxidase system, serial sections (2.5 nm) were

sliced, dewaxed, and rehydrated for hematoxylin-eosin (HE) staining or immunohistochemistry (IHC) as detailed previously. Viable tumor area per cross-sectional tumor area was performed using QuantCenter image analysis software (3DHISTECH), and tumor destruction ratio (TDR%) was assessed as described earlier.

5.5. Nanostring

100 ng RNA was used for hybridization. After hybridization, samples were transferred to the nCounter Prep Station for data collection on the nCounter Digital Analyzer. The 4.0 nSolver Analysis Software was used for data analysis. Genes with $\log_2$ fold change values greater than 1.5 or less than -1.5 were considered the most regulated for further analysis. Values obtained from three replicates of two groups, mEHT or mEHT+SC236, were used to generate the volcano plot. DEGs was conducted utilizing the Gene Ontology (GO) which was accessed through the DAVID. GO analysis was

used to identify genes that can be classified into different groups. In our study, we used the database for DAVID (https://david.ncifcrf.gov/) to perform functional annotation clustering the most regulated genes. The p-value represents the probability of chance association between genes and a specific functional category. The p-value was adjusted with the Benjamini-Hochberg procedure to control for false discovery rate (FDR) by correcting for multiple comparisons.

5.6. Statistical data analysis

The GraphPad Prism software was used for statistical analysis. Unpaired Mann-Whitney non-parametric tests were performed to compare the Sham and the mEHT-treated groups. Long term examinations were statistically evaluated with one-way ANOVA. Data are presented as mean ± SEM. Differences were considered statistically significant if $p < 0.05$.,

6. Results

6.1. mEHT induced IL-1β, IL-6, and COX-2 mRNA expression

mEHT induced the expression of pro-inflammatory cytokines (IL-6, IL-1β) in 4T1 TNBC *in vivo*. In the time-kinetic experiment, mice were terminated after the last mEHT treatment at 4h, 12h, 24h, 48h and 72h. IL-6 peaked at 12 hours ($p=0.007$), while IL-1β peaked at 24 hours after the last mEHT treatment ($p=0.009$). IL-6 was 3.8- and IL-1β 3 times higher in the mEHT-treated mice vs Sham. COX-2 mRNA was significantly elevated at 72h after the last mEHT session ($p=0.01$) and was increased even at 96 hours after 2X mEHT treatment.

6.2. mEHT disrupts blood vessels with following recovery seen in the endothelial marker CD105 expression in 4T1 TNBC

mEHT treatment significantly downregulated CD105 expression at 12h ($p=0.0002$) compared to

the Sham treated group, however, its expression return to the Sham level afterwards

6.3. mEHT induced expression of IL-1β, IL-6 and COX-2 was inhibited by NSAIDs.

In a separate experiment, mEHT and NSAID combination therapy was investigated. The mRNA level of IL-1β and COX-2 were upregulated 24h after the 4 mEHT treatments. IL-1β induction was almost completely reversed to the Sham level using SC236, but not aspirin, whereas COX-2 induction was reversed by both SC236 and Aspirin.

6.4. mEHT inhibited tumor growth was accelerated by NSAID co-treatment.

During the experiment of mEHT and NSAID combination therapy experiment tumor volumes were assessed by ultrasound. Tumor volumes progressed in Sham+vehicle treated mice from 264 ± 67 mm^3 (after 2 treatments) to 320 ± 59 mm^3 (after 3 treatments) and to 413 ± 77 mm^3 (after 4 mEHT treatments). Monotherapy mEHT +vehicle was able

to significantly reduce tumor size after 3 mEHT treatments (p=0.006). Average volume in the mEHT-treated group was 258 ± 54 mm^3 compared to 320 ± 59 mm^3 in the Sham-treated group. However, the combination therapy (mEHT+ASA and mEHT+SC236) was able to significantly reduce tumor volume already after 2 mEHT treatments to 156 ± 51 mm^3 (p=0.02) and 145 ± 49 mm^3, respectively, (p=0.02) compared to the Sham-treated group with an average volume of 264 ± 67 mm^3. After 4 treatments, only the COX-2 specific combination (mEHT+SC236) with 166 ± 54 mm^3 average volume proved to be significantly more effective than mEHT monotherapy (average volume 285 ± 77 mm^3). The COX-2 specific combination (mEHT+SC236) was the most effective inhibitor of tumor growth. This observation was supported by significant reduction of the tumor weight at the end of the study. Average tumor weight in the mEHT treated group was 284 ± 88 mg, while in the

mEHT+SC236 it was only 175 ± 51 mg (p=0.04). Bodyweight by the end of the study did not differ significantly from the initial bodyweight in any of the groups.

6.5. mEHT-induced tumor tissue destruction proved to be cC3-dependent apoptosis that was enhanced by NSAIDs

H&E staining was performed on tumor samples taken after the termination of the mEHT and NSAID combination therapy experiment. mEHT monotherapy (TDR=56 ± 5 %, p=0.9) or in combination with aspirin (TDR=66 ± 10 %, p=0.4) increased the TDR% to some extent, however, these did not reach statistical significance. Significant increase of the TDR was only achieved in the group treated with mEHT + SC236 (TDR=75 ± 14 %). The TDR in the mEHT + SC236 group was significantly different from the mEHT monotherapy group.

6.6. Multiplex (Nanostring) analysis demonstrated that COX-2 inhibition negatively correlated with tumor promoting factors associated with tumor cell membrane and extracellular matrix

Three samples from the mEHT and mEHT+NSAIDs experiment passed the Nanostring quality control (QC) and the Nanostring run was successful. 74 genes were identified as differentially expressed (DE) that are displayed on the heat map Genes were identified and clustered in two groups using DAVID. 7 genes were identified as membrane proteins and 6 genes as secreted proteins.

6.7. Aspirin diminished lung nodules in the B16F10 melanoma model

In the melanoma tail vein injection model pulmonary melanoma nodules were counted macroscopically. In untreated (Sham lungs 38.1 ± 16) nodules were counted. mEHT treatment alone reduced the number of nodules (28.8 ± 12) although

the difference was not statistically significant However, mEHT combined with aspirin, significantly decreased the number of focies compared to mEHT alone (8.1 ± 8). On the other hand, aspirin alone (31 ± 29) had no significant effect compared to Sham (26 ± 14).

7. Discussion

Triple-negative breast cancer (TNBC) is a leading cause of cancer mortality and lacks modern therapy options. Modulated electro-hyperthermia (mEHT) is an adjuvant therapy which demonstrated clinical efficacy for the treatment of various cancer types. In this study, we report that mEHT monotherapy stimulated interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) expression, and consequently cyclooxygenase 2 (COX-2), which may favor a cancer-promoting tumor microenvironment. Thus, we combined mEHT with non-steroid anti-inflammatory drugs (NSAIDs): a non-selective aspirin, or the selective COX-2 inhibitor SC236, *in*

vivo. We demonstrate that NSAIDs synergistically increased the tumor growth antagonizing effect of mEHT in the 4T1 TNBC model. Moreover, the strongest tumor destruction ratio (TDR) was observed mEHT was applied with the the combination of SC236. IL-1β and COX-2 expression were significantly reduced by the combination therapies. Furthermore, tumor damage was accompanied by a significant increase in cleaved caspase-3 (cC3), suggesting that apoptosis played an important role. Additionally, a custom-made Nanostring panel demonstrated significant upregulation of genes participating in the formation of the extracellular matrix and cell membrane functions. Similarly, in the B16F10 melanoma model, mEHT and aspirin synergistically reduced the number of melanoma nodules in the lungs. In conclusion, mEHT combined with selective COX-2 inhibitors may offer a new therapeutic option in the treatment of TNBC.

8. Conclusion

We suggest a new combination treatment protocol, which could be implicated in the clinical setting as a therapeutic option due to its effectiveness and availability.

We can drown the following conclusion based on our results:

1. mEHT treatment stimulated the expression of proinflammatory cytokines IL-1β, IL-6, and COX-2 in 4T1 TNBC
2. The combination therapy of mEHT+aspirin and mEHT+SC236 demonstrated a synergistic inhibition of tumor growth in 4T1 TNBC animal cancer model. However, selective COX-2 inhibition proved to be more effective
3. The mEHT induced expression of IL-1β and COX-2 were attenuated after COX-2 inhibition

4. Selective COX-2 inhibition enhanced mEHT-induced tumor destruction
5. Selective COX-2 inhibition may modulate the extracellular matrix, and cell membrane functions in the tumor microenvironment leading to inhibition of cancer cell proliferation
6. Apirin+mEHT demonstrated synergistic lung nodule inhibition in B16F10 melanoma mouse model

9. Bibliography of the candidate's publications

I. Publications used in the thesis

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