Potential therapeutic targets in melanoma: studies on NF-κB inhibitors and on the immunomodulating effect of melanoma-derived exosomes

Ph.D. thesis summary

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Introduction

According to the accepted model, many genetic changes are necessary to the malignant tumor formation. These genetic changes make tumor cells independent from growth factors, contact inhibition disappears, errors occur in programmed cell death, genetic stability is lost, resulting in a malignant phenotype characterized by angiogenesis, invasion and metastasis formation. Due to these genetic alterations, tumors will sooner or later be detectable by the immune system; therefore they have to modulate the immune system to survive.

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway plays a crucial role in cancer. Increased activity of the transcription factor was documented in many tumor types, which was related to different steps of tumorigenesis. The chemotherapeutic drugs can further increase the NF-κB activity that, in turn, can protect tumor cells from chemotherapy-induced cell death.

The tumor-host communication and the altered tumor microenvironment are crucial components of the tumor development, metastasis formation and chemotherapy resistance. Tumor-derived exosomes are essential mediators of this communication. Exosomes are microvesicules of 20-100 nm diameter. They can be produced by each mammalian cell types. The exosomes have endosomal origin, and they are released from the cells by exocytosis. Exosomes have diverse activities; they can influence the target cells by their protein, cell surface receptor, transcription factor, mRNA and miRNA content. The role of immune modulation exerted by tumor-derived exosome in tumorigenesis is still not completely clear. Activating and inhibitory effects have likewise been revealed, depending on the tumor developmental stage and the type of exosomes. The information that exosomes are carrying influences the migration of tumor cells, the antigen-specific T cell response and the polarity of the immune response.

The melanoma, a tumor that derives from melanocytes, is a malignant and invasive skin cancer. Melanoma forms metastases by high probability. Its genetic variability is higher than most other tumors, and it effectively avoids immune surveillance. The exact mechanism of this immune escape is unclear. One mechanism might be the constitutive NF-κB activation leading to induction of anti-apoptotic, pro-angiogenic and metastasis promoting proteins. Exosomes produced by the tumor might also be involved in the process. The tumor derived exosomes can alter the tumor stroma, which can contribute to avoiding immune surveillance. Importantly, the chemotherapy induced NF-κB activity increase can further enhance exosome release.

Aims of the study

Since the NF-κB signal pathway plays a complex role in the tumor formation – it increases the invasivity and metastatic potential of the tumor, furthermore, it is one of the major mediators of chemotherapy resistance –, in the first part of my work I was investigating potential NF-κB inhibitors. Our aims were:

- To test the inhibitory effect of vanillin and nine related aromatic aldehydes on human melanoma cell proliferation and NF-κB activity.
- To investigate the impact of the aldehydes alone or in combination with two chemotherapeutic agents, doxorubicin and cyclophosphamide, on the cell viability and NF-κB activity in melanoma cells.
- To explore the effect of the most promising aldehydes in a melanoma xenograft model *in vivo*.

Tumor-host communication and the alternated tumor microenvironment are important factors of tumor formation, metastasis and chemotherapy resistance. Its important mediators are the tumor derived exosomes. In the second part of my work we investigated the impact of tumor derived exosomes in tumor-host communication. Our aims were:

- To develop a standard protocol for exosome isolation and characterize the purified exosomes.
- To examine the influence of exosomes on immune cells, i.e., dendritic cells, T cells and macrophages.

Methods

- XTT cell proliferation assay for assessing the effect of vanillins and chemotherapeutic agents on the A375 human melanoma cell viability
- For examining the NF-κB signaling pathway, we used an NF-κB-Luc. melanoma reporter cell line and luciferase assay
- We used an NSG melanoma xenograft model for assessing the *in vivo* impact of vanillins on tumor growth
- The exosome characterization was performed by atomic-force microscopy and transmission electron microscopy
- Thymidine incorporation assay was used for examining the effect of exosomes on dendritic cell maturation, as measured by dendritic cell induced T cell proliferation

- The cytokine and chemokine profile of exosome treated macrophages was determined with a multi-dot blot Proteome Profiler Array

Results

In the first part of the study we investigated the impact of vanillin and/or doxorubicin on cell proliferation and NF-κB signaling using the A375/NF-κB.Luc.4 (neo) reporter cell line. Six of the ten tested aldehydes had cytotoxic effect: 2,4,6-Trymethoxybenzaldehyde, 2,5-Dimethoxybenzaldehyde, 2-Nitrobenzaldehyde, 2,4,6-Trihydroxybenzaldehide (TBA), *ortho*-Vanillin, 3-Quinolinecarboxaldehyde. TBA and *o*-vanillin were the most active. Doxorubicin activated the NK-κB signal pathway of the A375 cells. This activity was reduced by 2,4,6-Trymethoxybenzaldehyde, 2,4-Dihidroxybenzaldehide, 2-Nitrobenzaldehyde, *ortho*-Vanillin, TBA and 3-Quinolinecarboxaldehyde treatment. *o*-Vanillin appeared to be the most active. *o*-vanillin reduced the doxorubicin induced NF-κB activity and the basal (constitutive) activity of the melanoma cells as well.

Then we examined the inhibitory effect of *o*-vanillin and TBA on NF-κB activity induced by another chemotherapeutic agent, the alkylating compound cyclophosphamide. 4-hydroperoxycyclophosphamide (4-HC), a bioactive derivative of cyclophosphamide, used at 12.5 μM concentration, increased the NF-κB activity by 50%. *o*-Vanillin was suppressed this increased activity by 43%, while TBA by 20%.

Based on the *in vitro* findings, *o*-vanillin and TBA were selected for *in vivo* efficacy test in A375 human melanoma-bearing NSG mice, as a single agent and in combination with cyclophosphamide. The growth inhibition of the *o*-vanillin/cyclophosphamide combination reached statistical significance by day 15, and remained statistically significant until the end of the experiment (*i.e.* day 20). Moreover, on day 20, the antitumor effect of both tested aldehydes, as single agents, was significant.

With these experiments we showed that the *o*-vanillin and TBA have anti-tumor effect *in vitro* and *in vivo* as well.

In the second part of the work, we were investigated the immunomodulatory effect of exosomes in the well known B16F1 mouse melanoma model. After multiple differential filtrations the exosomes were isolated with ultracentrifugation. Size and form distribution of exosomes was determined by atomic force microscopy. Our results were in accordance with data from the literature, as we purified exosomes in the range of 20-100 nm in diameter.

Transmission electron microscopy showed that they had no internal structure, excluding viral contamination.

Then we showed that melanoma cell derived exosomes promoted the maturation of dendritic cells, as the treated dendritic cells induced more intensive T cell proliferation in a co-culture system. These results are in contrast with several earlier papers suggesting that exosomes suppress, rather than promote dendritic cell maturation, therefore they might not enhance anticancer immunity. On the other hand, there are publications that report that exosomes might induce anti-tumor immunity. In some models, tumor cell derived exosomes carrying tumor antigens can activate the dendritic cells that, in turn, activate antigen specific cytotoxic T cell response. For example, exosomes originating from Hsp70/Bag-4 membrane-positive pancreas- and colon tumor cells stimulate migration and reactivity of NK cells. In our experiments, exosomes induced NF-κB activation in macrophages.

The cytokine and chemokine expression changes induced by the exosomes reflect an alternatively activated macrophage profile. The exosome induction resulted in a cytokine and chemokine profile different both from the M1 and the M2 profiles. The reduced level of TIMP1 matrix metalloproteinase inhibitor might be involved in metastasis formation. The decreased level of IFNγ and IL-16 might reflect a reduced anti-tumor M1 response, together with the increased level of IL-1Ra and IL-13, both well known suppressors of the type 1 immune response. CCL2, IL-8 and MIP-1 were expressed in high levels in our model. They have been shown to be involved in inflammation, angiogenesis, and tumorigenesis and wound healing. The elevated level of MIP-1 and IL-8 is correlated with the constitutive activation of the NF-κB in melanoma cells. Because TNF-α and other anti-tumor cytokines were also present in our experiments, we cannot set up a clearly defined tumor supporting profile either.

Taken together, B16F1 melanoma cell derived exosomes can influence the immune response. In *in vitro* conditions the exosomes alter dendritic cell and macrophage function, induce T cell proliferation and NF-κB activation. The exosome treatment changes the cytokine and chemokine profile of the macrophages, resulting in the expression of both tumor promoting and anti-tumor cytokines and chemokines.

The conclusion of our observations is that exosomes are immunologically active participants of the tumor-host communication. Due to numerous factors, tumor microenvironment is characterized by type 2 immune polarization. In our *in vitro* experiments, the exosome-treated macrophages show a „mixed” polarization profile, in the tumor microenvironment, exosomes are likely to enhance the existing type 2 polarization. In

summary, interacting with other elements of the tumors microenvironment, exosomes play an important role in tumorigenesis.

Conclusion and final remarks

From the tested aldehydes, *ortho*-vanillin and 2,4,6-trihidroxybenzaldehyde significantly reduced melanoma cell growth. The selected vanillins reduced the basal- and chemotherapy induced NF-κB activity of melanoma cells as well. In an *in vivo* mouse xenograft model, *ortho*-vanillin and 2,4,6-trihydroxybenzaldehyde inhibited primary tumor growth as monotherapy, or in combination with cyclophosphamide. According to our results, these aldehydes might be considered for use as adjuvant therapy in melanoma.

Melanoma cell derived exosomes promoted the maturation of dendritic cells, resulting in enhanced dendritic cell-induced T cell proliferation. The exosomes induced macrophage activation, and altered the macrophage cytokine and chemokine profile that was different from both the LPS and the IL-4 induced profiles. Since the melanoma cell derived exosomes have a unique and complex immunomodulatory effect. Based on our results, they might be considered as diagnostic markers and therapeutic targets.

List of publications

MTMT identifier: 10032800

The thesis was based on the following publications:

Marton A, Kúsz E, Kolozsi C, Tubak V, Zagotto G, Buzás K, Quintieri L, Vizler C. Vanillin Analogues o-Vanillin and 2,4,6-Trihydroxybenzaldehyde Inhibit NFκB Activation and Suppress Growth of A375 Human Melanoma. *Anticancer Res, in press* IF=1,826

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Other publications

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