SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The roles of TASK-3 channels and muscarinic cholinergic receptors in melanoma cells

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UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2015

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The Examination takes place at the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, 2015.04.09. 11:00

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Introduction

The melanoma

The malignant melanoma (often called simply melanoma) is a tumour which, in most of the cases, develops as the malignant transformation of the skin-situated melanocytes. Although extracutaneous melanomas also exist (e.g., colorectal, retinal, conjunctival, or vaginal melanoma), their incidence is far smaller than that of the skin-originated version: the incidence of the cutaneous melanomas is 40–50/100,000, whereas the extracutaneous melanomas form only 4–5% of all melanoma cases.

Due to their most common localisation, melanomas are compared statistically usually to other skin tumours. These analyses highlight the dire prognosis of melanoma: even though it is responsible for only 4% of all skin-related tumours, it accounts for 65% of the death toll of all skin tumours. Although studies identified several risk factors of the formation of melanomas (e.g., high number of naevi), there is concordance among the experts that the most important trigger of melanoma formation is the ultraviolet radiation during sun exposure.

As the proliferation rate of the melanoma cells is high, the most important aspects in the therapy are early diagnosis and the fastest possible surgical removal of the tumour. Unfortunately, the removal of the primary tumour does not necessarily mean definitive treatment of the disease, because early metastasis formation is a significant factor in determining the malignancy of the melanomas.

Malignant melanoma is a heavy economical and healthcare burden in most countries. It is not surprising, therefore, that there is intense research to reveal the pathomechanism of the disease and to develop potential therapies. The relevant studies focus on modifying the high proliferation rate and the early metastasis formation of the melanomas.

Programmed cell death

Under physiological conditions cell division and cell death together assure the continuous renewal of a cell population and its constant cell number. One of the physiological forms of cell death is apoptosis, which is the opposite of mitosis in controlling the cell population. During tumour formation the fine balance between cell division and cell death is lost. An increase rate of cell division and a decreased rate of cell death together are responsible for the upset of the balance. Due to the fact that numerous forms of cell death have been identified, the term ‘programmed cell death’ has been introduced. This term refers to various intracellular processes in which cells stop their function and die according to a genetically determined program.

One of the thoroughly studied cell death forms is apoptosis. This mechanism is a precisely controlled, ATP-dependent process, in which no inflammatory response occurs during the cell destruction. The apoptosis starts with the decrease of cell volume, followed by the condensation and fragmentation of the cell nucleus, blebbing, and finally loss of the adhesive connections to the neighbouring cells occurs. The apoptotic process can be initiated by external signals; in this case the activation of the extrinsic pathway leads to cell death. In other instances apoptosis can be triggered by the activation of the intrinsic pathways that may lead to either caspase-dependent or caspase-independent processes.

The extrinsic apoptotic cascade is activated by the 'death receptors' (DR), whereas activation of the intrinsic (or mitochondrial) apoptotic cascade is initiated by DNA damage. In the intrinsic pathway caspase-9, whereas in the extrinsic pathway caspase-8 is responsible for the activation of the effector caspases (3, 6, and 7). The cell nucleus condensation and the cell volume decrease that both accompany apoptosis are the results of the activity of the effector caspases. During intrinsic apoptosis, in addition to the caspase-dependent processes, caspase-independent steps also contribute to cell death. Among the latter ones, AIF (apoptosis inducing factor) is a key molecule. The regulation of the apoptotic processes is a complicated scheme, which includes several molecules. Among others, the BcL (B cell lymphoma) molecule family is a regulatory factor that consists of both pro-apoptotic and anti-apoptotic proteins.

Considering the significance of apoptosis in controlling cell number, the detection of apoptosis has a great importance in tumour research. The annexin V molecule efficiently binds to the phosphatidyl serine situated on the outer side of the plasma membrane. Consequently, the accumulation of annexin V in the outer side of the cell membrane reliably shows the activation of the apoptotic cascade. Another way of apoptosis detection is the visualisation of DNA fragmentation, for which the TUNEL assay is the most common method. Moreover, the mitochondrial dysfunction accompanying the apoptosis can also be detected by monitoring the mitochondrial membrane potential using fluorescent dyes (e.g., DiLc, RHOD-123, JC-1, or DioC6).

According to the widely accepted view, necrosis is also a form of programmed cell death, although it has been earlier considered as a spontaneous process. As the final result of the necrosis is the irreversible rupture of the cell membrane, the more appropriate term for this phenomenon is oncosis or oncosis-necrosis. The initial step of the necrosis is the entry of a toxic material into the cell that inhibits ATP production.

Metastasis formation

Besides the increased cell division rate, other characteristic features of the malignant tumours are the infiltration of the neighbouring tissues and the formation of metastases. The initial step of the tumour cell migration is focal adhesion. This is a complex process during which the components of the cytoskeleton and the extracellular matrix interact by utilizing various signal transduction pathways. Initially the primer tumour grows by infiltrating the neighbouring tissues, and then the malignant cells invade the blood vessels. These cells travel with the circulation and in distant parts of the body they leave the blood-stream and form micrometastases. In the new environment the proliferation program of the cells is transitionally suspended in order to adapt to the new surroundings. After the adaptation, the cell division program is activated again, and clinically detectable macrometastases are formed.

Many recently described intracellular signalling cascades that are based on chains of specific protein-protein interactions play important roles in tumourgenesis. These cascades are known to play important roles in controlling physiological cell functions. However, under some still unknown conditions the same regulatory pathways may contribute to changing the physiological functions to pathological ones (such as tumourgenesis). An obvious question that needs to be asked is whether similarly to the newly described signalling cascades, the well-known 'classical' pathways also have roles in tumourgenesis.

Potassium channels

The different ions influence many cell functions. This modification may be direct or indirect; in the latter case ions usually modulate the membrane potential. The electrical properties of the cell membranes are set by the heterogeneity of the expressed ion channels, among which the highest heterogeneity is presented by the potassium channels.

The generally accepted categorisation of the potassium channels distinguishes four families. These are (i) the calcium-activated potassium channels (BK and SK types), (ii) the inward rectifier potassium channels (Kir subtypes), (iii) the voltage-gated potassium channels (Kv subtypes), and (iv) the two-pore-domain potassium channels (K2P subtypes).

The potassium channels have many different physiological functions. Due to its importance in the history of biological sciences, it must be mentioned that the Kv channels play important roles in determining the time course of the action potential (AP). From the aspects of tumourgenesis it is vital to emphasize that the potassium channels may play roles in regulating both the cell proliferation and the programmed cell death. Several bodies of evidence suggest that the opening of potassium channels in the cell

membrane causes hyperpolarisation facilitating, therefore, the entry of calcium ions into the cytosol and increasing the proliferation rate. This mechanism was suggested in the cases of lymphocytes and melanoma cells.

The role of the potassium channels in regulating the cell cycle is further complicated by the fact that some of these channels are also localised in the mitochondria. There is consensus in the literature that these channels can alter mitochondrial functions by influencing the mitochondrial membrane potential (MMP). The controversial role of the mitochondrial potassium channels is best illustrated by the fact that both their opening and closing can initiate apoptosis.

In the recent years several observations have been made about the role of the twin-pore TASK-3 potassium channels in tumourgenesis. Some of these results favour the tumour-promoting effect of these channels. The explanation of this effect may be the inhibition of apoptosis. It is notable that only the functional (potassium permeable) TASK-3 channels have this property, suggesting that the tumour-promoting effect is somehow related to the movement of potassium ions through the channels. Previous results of our laboratory also suggested that the inhibition of TASK-3 expression in melanoma cells damages the mitochondrial function and eventually kills melanoma cells.

Muscarinic receptors and calcium homeostasis

The cytoplasmic calcium ion concentration is a main element of many common signal transduction pathways. The elevation of this concentration is the trigger, which can change the activity of several intracellular processes.

The cytoplasmic calcium level can only serve as an efficient modulator if its elevation is followed by rapid normalisation. This decrease can be temporarily achieved by the calcium binding buffer proteins (such as parvalbumin, calbindin, or calretinin). In a long term, however, removal of the calcium from the cytosol must be ensured. One option is to pump the calcium ions into the extracellular space, whereas another option is to transport the calcium ions back into the intracellular stores. The significance of calcium ions in modulating several cell functions is well known. The two most thoroughly investigated fields are muscle contraction and neurotransmitter release. The calcium ions can act in the cell nuclei, too, where they can induce gene expression changes and alter the cell cycle.

According to the literature there is no such component of the calcium homeostasis of the malignant cells which is specifically present only in them. In other words, the tumour cells use the same mechanisms to regulate their calcium homeostasis as the healthy cells—but with different biological purposes. In the tumour cells several components of the calcium

homeostasis contribute to the activity of RAS and its signal transduction pathway to promote cell proliferation. Though our present knowledge is not enough to assemble the relevant experimental results into a comprehensive theory, there is agreement that calcium ions have significant roles in regulating the survival, proliferation, migration, and metastasis formation of melanoma cells.

The importance of the muscarinic cholinergic receptors has been described in several types of tumour cells. It has been established, for example, that in different lung tumour-originated cell lines the MR3 increases the activity of MAPK (mitogen-activated protein kinase) cascade and, consequently, the proliferation rate of the cells.

In studies performed to understand the highly invasive character of malignant melanoma, it was discovered that melanoma cells express several components of the muscarinic cholinergic transduction pathways. Considering the fact that during embryogenesis the muscarinic signal transduction plays important roles in the migration of the melanocyte precursor cells, it is reasonable to assume that MRs and their signal transduction pathways may also play roles in determining the behaviour of melanoma cells.

Although the muscarinic receptor—calcium homeostasis pathway plays important roles in the determination of the biological properties of tumour cells (including melanoma cells), there is no agreement regarding the identity of specific cholinergic effects exerted on melanoma cells.

Aims

According to the earlier results of our research group, TASK-3 channels play a role in maintaining the mitochondrial function of malignant melanoma cells. For easing further investigation of this role, we stably gene silenced the TASK-3 protein expression in the primer tumour-related WM35 melanoma cell line. It has been demonstrated, however, that WM35 cells are more responsive to therapeutic interventions than several other, clinically more relevant cell lines. For this reason, the more aggressive, lymph node-originated A2058 melanoma cells were also studied in the present work. A2058 cells were transiently transfected with the specific shRNA sequence. Based on our previous results, one of the main aims of the present work was to determine if reduced TASK-3 expression had any appreciable effect on the mitochondrial membrane potential. Moreover, determination of possible modifications in several other parameters, such as cell number, viability, and cell morphology was carried out, too.

Another aim of the present work was to study the muscarinic cholinergic effects on the calcium homeostasis in various melanoma cell types. Because preliminary data proved that the cholinergic agonist carbamyl-choline evoked calcium transients in cultured melanoma cells, further specific experiments were carried out using this agonist. The aims of this part of the work were to determine the sensitivity of melanoma cells to carbamyl-choline, to study the time course of the calcium transients, and to determine the source of calcium needed for these transients. An important aspect of the experiments was to compare the behaviour of the primary tumour-originated WM35 cells and that of the metastatic HT168-M1 and HT199 cells.

Materials and Methods

Cell culturing

Four human melanoma cell lines (WM35, A2058, HT199 and HT168-M1) were used for the present work. The WM35 was considered as a model representing primary tumours, while the other three cell lines (A2058, HT199 and HT168-M1) were regarded as metastatic melanoma models. Culturing of these cells was done in RPMI 1640 medium supplemented with necessary additives. Monolayer cultures were kept in a 5% CO_2 atmosphere at 37°C.

Generation of TASK-3 gene silenced melanoma cells

An effective TASK-3 shRNA plasmid construct was designed and tested as described earlier by our research group. A scrambled sequence which did not interfere with any human protein expression sequence was used as control. The inserts were ligated into BamHI- and HindIII-digested pRNATH1.1/Neo expression vector. The TASK-3-specific and scrambled shRNA vector constructs were transfected into WM35 and A2058 melanoma cells using Lipofectamine 2000 transfection reagent, according to the manufacturer's instructions.

RT-PCR

Total RNA was isolated from transfected and non-transfected melanoma cell cultures. The reverse transcription reaction was carried out with Omniscript RT Kit. Amplifications of the TASK-3- and β-actin-(internal control) specific cDNA sequences were achieved using specific primers designed by our group. PCR reactions were carried out in a programmable thermocycler. PCR products were mixed with EZVision DNA staining kit, followed by electrophoresis of the amplified products, which was performed on agarose gels. Images were taken from the gels using ultraviolet light in a Fujifilm Labs-3000 dark box. Densitometry was performed with ImageJ.

Western blotting

Total cell lysates isolated from non-transfected and transfected melanoma cell cultures were prepared using lysis buffer. The amount of protein in the samples was determined by the Pierce BCA Protein Assay Kit. Samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with the primary antibodies. After washing, the membranes were incubated with the appropriate secondary antibody. The immunoreactive bands were visualised using an enhanced chemiluminescence Western blotting Pico or Femto detection kit in a Fujifilm Labs-3000 dark box.

Assessment of the mitochondrial membrane potential

In some experiments MMP was studied using the fluorescent dye JC-1. JC-1 permits discrimination between energised (polarised) and de-energised (depolarised) mitochondria because the green-emitting monomeric dye molecules form aggregates (J-aggregates) and present red fluorescence inside the highly polarised mitochondria. When excited at 514 nm, the monomeric form of JC-1 emits fluorescence best detected at 530 nm. In contrast, J-aggregates emit fluorescence with a maximum of 595 nm. Melanoma cells on coverslips were incubated with JC-1 then kept in dye-free RPMI-1640 solution. The fluorescence measurements were conducted using a Zeiss LSM 510 Meta confocal microscope. The background-corrected green and red fluorescence intensities were determined and MMP was approximated by the background-corrected red/green fluorescence intensity ratios.

In a different set of experiments, MMP was monitored using DiOC6. At the end of the incubation, melanoma cells were washed with PBS, trypsinized, and analysed with a BD FACS Calibur flow cytometer.

Time-dependent changes of MMP were studied as modifications in the fluorescence intensity of the JC-1 aggregates using an upright microscope equipped with a fluorescence imaging system. A specific emission filter in combination with a dichroic mirror was employed. The excitation wavelength was set to 561 nm and the emission was detected at 590 nm. In the appropriate experiments mitochondrial depolarization was evoked by [(3-chlorophenyl)hydrazono]malononitrile (CCCP).

MTT viability and proliferation assay

Cells were seeded into 96-well plates. On the first and third day of culturing the wells were washed with calcium- and magnesium-free PBS (CMF-PBS) then incubated with MTT reagent. The produced formazane content of the medium was assessed at 550 nm using a Model 550 Microplate Reader.

Determination of the amount of mitochondrial DNA

Total RNA was isolated from transfected and non-transfected melanoma cell cultures. The reverse transcription reaction was carried out with Omniscript RT Kit. Mitochondrial and nuclear DNA was amplified in qPCR using a Light-Cycler 480 System and Maxima SYBR Green/ROX qPCR Master Mix with specific primers. Quantification of the number of mitochondria was carried out by using the ratio of the mitochondrial and the nuclear DNA contents (H2/H1 ratio).

Intracellular calcium imaging

To measure intracellular Ca^{2+} at single cell level, the fluorescent dye Fluo-4 was used. The cells seeded onto coverslips were incubated first with the dye (dissolved in Hanks' solution), then with dye-free Hanks' solution. The Ca^{2+} imaging system contained various units which were produced by Till Photonics and Zeiss. Some components were chosen according to the optical properties of Fluo-4. Excitation was achieved at 494 nm, and the calcium transients were evoked by the application of carbamil-choline (CCh).

To determine the dose-response relationship of the CCh effect, cells were seeded into each well of a Greiner black 96-well plate in another set of experiments. The melanoma cells in the Greiner plates were loaded with fluo-4-AM. The excitation and emission maxima were set to 495 and 520 nm, respectively (Flex Station II 384 high throughput screening system). The individual data points were obtained as the maximum fluorescence intensities recorded during the incubation with various CCh concentrations.

Determination of the proportion of cells committed to apoptosis or necrosis

Melanoma cells were seeded onto glass coverslips and cultured there. Cells were washed with PBS then incubated with fluorescein isothiocyanate (FITC)-conjugated annexin-V and propidium iodide to assess the rates of apoptosis and necrosis, respectively. After incubation, cells were washed with PBS and mounted with Mowiol without fixation. Annexin-V- and propidium iodide-specific fluorescence signals were detected using a laser scanning confocal microscope. Excitation and emission wavelengths were 488 and 505–550 nm (Annexin-V) and 543 and 560–590 nm (propidium iodide), respectively.

Isolation and Western blotting of mitochondrial and cytosolic fractions

Cells maintained in tissue culture flasks (TPP) were gently trypsinised. Cell suspension was collected and centrifuged at 600g using a Heraus Biofuge Stratos Centrifuge. The supernatant was discarded and the pellet, which contained the cells, was re-suspended in IBC (isolation buffer for cells). The suspension was transferred to a Teflon potter and homogenised. The homogenised solution was centrifuged at 600g. The supernatant was carried over to fresh tubes, whereas the cell nuclei-rich pellet was re-suspended in protease inhibitor-containing lysis buffer. The supernatant was centrifuged at 10,000g. The resulting supernatant containing the cytosolic fraction and the mitochondrium-rich pellet were prepared for Western blot experiments. Detection of cytochrome c in the mitochondrium-rich and cytosolic fractions was carried out using Western blot experiments.

Caspase 3 assay

Caspase 3 activity was measured using a Caspase 3/CPP32 colorimetric protease assay, according to the manufacturer's instructions. Caspase 3 activity was assessed by measuring the absorbance of each sample at 370 nm (FlexStation3). In positive controls, apoptosis was induced by incubating the cells with H_2O_2.

CyQUANT_GR cell proliferation assay

For the CyQUANT GR assay, cells were seeded into each well of a Greiner 96-well plate. On the third day, cells were washed with CMF-PBS, kept at -70^{o}C, and incubated with DNA-staining solution. The fluorescence intensity was measured in a FlexStation3® device using 485 nm and 530 nm as excitation and emission wavelengths, respectively.

Fluorescent immunocytochemistry

The cells were first fixed with paraformaldehyde, and then washed with PBS containing glycine. Permeabilisation was performed by incubating the cells in PBS (containing Triton X-100). To prevent non-specific binding, the cells were kept in PBS (containing bovine serum albumin). In the next step the melanoma cells were incubated with the primary antibodies then rinsed in PBS. After the application of the primary antibodies the cells were incubated with the appropriate secondary antibody. Finally, the nuclei were stained with 4',6-diamidino-2-fenilindol (DAPI) and the cells were mounted onto a coverslip.

Mitochondrial labelling was achieved using Mitotracker Red (CMXRos probe). Melanoma cells were incubated with MitoTracker-Red containing medium, washed with CMF-PBS, and fixed with paraformaldehyde. Cells were in all cases investigated using a Zeiss laser scanning confocal microscope.

Assessing cell shapes and determination of cell size

Morphological classification of the cells was based on their shape and number of processes. WM35 melanoma cells were seeded onto coverslips and cultured there, and then the cells were fixed with paraformaldehyde, and bright-field images were captured using the Till Photonics imaging setup. Melanoma cells were defined and their areas were determined using ImageJ.

Statistical analysis

Results are given as mean ± SEM throughout. Statistical difference was assessed by ANOVA and Tukey–Kramer post hoc and Students T-test test. For calculations and for constructing plots, the Origin v8 and Microsoft Excel programs were used.

Results

The most convenient way to identify the role of the TASK-3 channels in melanoma cells would be the inhibition of these channels. At the beginning of the experiments, however, no specific inhibitor of this channel type was known. To overcome this difficulty, WM35 melanoma cells were transiently transfected with an shRNA sequence. As a result of this intervention, the TASK-3 expression of the affected melanoma cells was significantly reduced. In the present study, the first task was to produce a TASK-3 gene silenced cell culture with higher transfection rate, which can be used for longer periods. To achieve this, a stably TASK-3 gene silenced cell line was developed. The key step in producing such a cell line was the elimination of the non-transfected cells with geneticin, and culturing the surviving transfected, geneticin-resistant cells.

The WM35 melanoma cell line was derived from a superficial skin melanoma that was in its radial or early vertical-growth phase. It has been demonstrated that the WM35 cells are more responsive to cancer treatment than several other, clinically more relevant cell lines. The WM35 cells alone, therefore, might not be ideal subjects of studies with possible therapeutic relevance. For this reason, some of the experiments were repeated using the more aggressive A2058 melanoma cells.

As the first step, we established that—similarly to their WM35 counterparts—A2058 cells express TASK-3 channels, too. The presence of TASK-3 in A2058 cells was demonstrated by immunocytochemistry. Unlike WM35 cells, the A2058 cells were transiently transfected with the specific sequence. This technique yielded enough cells for the experiments and, in the same time, helped to avoid any possible complication arising from the use of stable transfection (e.g., bias introduced by the clone selection). As expected, the transient transfection of the A2058 cells significantly reduced their TASK-3-specific mRNA and TASK-3 protein contents, as evidenced by the results of the relevant RT-PCR and Western blot experiments that were performed 4 days after the transfection.

One of the primary aims of the present work was to support and broaden previous observations indicating dysfunction of the transiently transfected WM35 cells. In the course of this work we turned our attention to the characterisation of the mitochondrial membrane potential (MMP). To achieve this, mitochondrial function was monitored by applying fluorescent dyes. One of these dyes was the JC-1. On using appropriate excitation wavelength, the monomeric dye emits green, whereas the J-aggregate form emits red fluorescence. When incubating the cells with JC-1, the monomeric dye molecules enter the cytoplasm then the mitochondria. The mitochondrial uptake, however, depends on the polarisation of the mitochondria. Mitochondria having high MMP accumulate JC-1 in large quantities in their

matrix, where the dye molecules form aggregates and emit red light. Consequently, cells possessing active mitochondria present high red/green JC-1-specific fluorescence intensity ratios. In contrast, mitochondrial depolarization is indicated by a reduced red/green fluorescence intensity ratio.

When loaded with JC-1, the TASK-3 knockdown WM35 cells presented spectral features that were different both from their scrambled shRNA-transfected counterparts and from the control cells. Namely, the knockdown cells presented appreciably weaker red and somewhat stronger green fluorescence than either of the other two cell lines. The red/green fluorescence intensity ratios were calculated and this parameter proved to be significantly smaller in the knockdown cells (0.77 ± 0.03; $n = 49$; $p < 10^{-4}$) than either in the scrambled shRNA-transfected (1.75 ± 0.11; $n = 37$) or in the control melanoma cells (2.31 ± 0.10; $n = 32$). Similarly to the WM35 cells, the knockdown A2058 melanoma cells presented significantly lower red/green fluorescence intensity ratio than their control counterparts (0.93 ± 0.07 versus 2.1 ± 0.2; $n = 25$; $p < 10^{-7}$). These results indicate that the mitochondria are depolarized in those melanoma cells whose TASK-3 expression has been reduced. Besides determining the red/green fluorescence ratio, the dye content of the WM35 cells was also approximated. Our aim was to highlight the differences in the amount of JC-1 present in the cells, which may point toward alterations in the dye-uptake and/or dye-removal characteristics of the three cell lines.

Confirmation of the above results was sought and obtained using another dye whose fluorescence properties also depend on MMP. It was established that the mean DiOC6 fluorescence intensity of the knockdown WM35 cells was about half of that produced by the control and scrambled shRNA-transfected cells.

Mitotracker is yet another fluorescent dye whose mitochondrial accumulation and light emission depend on the MMP. When cells were incubated with Mitotracker Red, the Mitotracker labelling intensity of the TASK-3 knockdown cell line was significantly lower than that of either the control or the scrambled shRNA-transfected WM35 cells ($p < 10^{-6}$ in both cases). Taken together, all results obtained by applying fluorescent dyes are coherent. These results indicate that the inhibition of TASK-3 expression results in depolarisation of the mitochondria and damages the mitochondrial function in melanoma cells.

In the next step of the experiments the apparent mitochondrial dysfunction was studied using an uncoupler compound to evoke mitochondrial membrane potential changes. On applying 50 μM CCCP in the extracellular medium, the red fluorescence intensity reduced in all cell lines tested. This decrease shows that (i) the mitochondrial uncoupling made the

mitochondria depolarised, (ii) the dye molecules moved back to the cytoplasm from the mitochondrial matrix, consequently (iii) the dye concentration in the mitochondrial matrix was reduced, and (iv) the previously formed JC-1 aggregates dissociated that resulted in (v) a decrease of the emitted red light. When the amplitudes of the CCCP-evoked light intensity changes were compared, no significant difference could be noted between the control and the scrambled shRNA-treated cell lines. In the knockdown cells, however, the reduction of the red fluorescence intensity was significantly smaller; approximately one-fourth of that observed in either of the other cell lines.

We have demonstrated that the TASK-3 knockdown cells accumulated the JC-1 dye to a smaller extent. In harmony with this phenomenon, the resting fluorescence intensity of the knockdown cells was smaller. Taking this difference into consideration, it is reasonable to suggest that in the case of the knockdown cells the smaller CCCP-evoked fluorescence intensity decrease might have been simply the consequence of the smaller resting fluorescence intensity in these cells. In order to clarify the situation, the size of the CCCP-evoked fluorescence decreases was normalised to the appropriate resting fluorescence intensities measured before the application of the uncoupler. These calculations revealed that while the relative fluorescence changes produced by the control and scrambled shRNA-transfected cells were very similar ($9.0 \pm 0.5\%$; $n = 76$ and $9.2 \pm 0.4\%$; $n = 77$, respectively), the knockdown cells produced an approximately 30% smaller relative fluorescence decrease ($6.12 \pm 0.02\%$, $n = 67$).

Because adequate mitochondrial function is essential for both the survival and the proliferation of cells, we assumed that the knockdown cells possessing depolarised mitochondria had reduced metabolic activity and decreased rate of cell proliferation. To confirm this, metabolic activity was assessed by MTT assay. It was found that the metabolic activity of the knockdown cells was significantly less than that of the control or scrambled shRNA-treated cells in both the WM35 and A2058 cultures.

Both the reduced Mitotracker labelling intensity of the knockdown cells and their reduced metabolic activity implied that TASK-3 knockdown cells might have fewer mitochondria. To explore this, the amount of mitochondrial DNA was also determined. It was established that the H2/H1 ratio was significantly lower in the knockdown than in the control or scrambled shRNA-transfected cells. We conclude, therefore, that reduced TASK-3 expression results in mitochondrial depolarization and decreased number of mitochondria.

Prominent differences could be noted in the morphology of the TASK-3 knockdown WM35 cells when compared to their healthy counterparts. First of all, the cell density of the TASK-3 knockdown cell cultures was reduced. Moreover, the shape of the individual melanoma cells was also different: in the knockdown cell culture about 58% of the cells presented elongated, fusiform appearance. In contrast, in the control and scrambled shRNA-treated cell lines the fusiform cell shape was the least common (accounting for only 12–13% of the cells). On studying cell morphology it was noted that besides affecting the shape of the cells, the reduced TASK-3 expression decreased the surface area of the cells as well. While the cells in the TASK-3 gene silenced cultures had an average surface area of $612 \pm 39\ \mu m^2$ ($n = 50$; $p < 0.05$), this value of the control and the scrambled shRNA-treated WM35 cells was bigger and similar to each other (736 ± 23 and $762 \pm 33\ \mu m^2$, respectively; $n = 50$ in both groups).

The previous results confirmed that in TASK-3 knockdown cultures the number of cells is reduced compared to control and scrambled shRNA transfected cell cultures. In our further experiments we investigated the processes responsible for this phenomenon. Based on the well-known connection between TASK-3 channel activity and cell apoptosis, we studied if reduced TASK-3 expression has any effect on the apoptotic activity. Because the cell number in the cultures might also be affected by necrosis, we investigated the intensity of necrosis as well. As expected, the proportion of cells committed to apoptosis was very small in the control WM35 melanoma cell culture (< 5%). Transfection with a scrambled RNA sequence did not induce apoptosis either. In contrast, TASK-3 knockdown cells demonstrated a massively increased annexin-V labelling: labelling could be observed in about 50% of the cells. In contrast, decreased TASK-3 protein expression of the WM35 melanoma cells did not promote necrotic cell death: propidium iodide labelling of the cells could be detected only in the positive control experiments.

Further evidence supporting the causative link between TASK-3 gene silencing and activation of apoptotic mechanism was gathered by comparing the amount of cytochrome c protein in mitochondrium-rich and cytosolic fractions prepared from all cell lines. The relative cytochrome c protein content of the cytosolic fraction was high in the knockdown cells, whereas it was hardly detectable in either the control or the scrambled shRNA-transfected cultures. An opposite trend was seen in the mitochondrium-rich fraction: in this case the knockdown cells presented low relative cytochrome c densities, whereas the cytochrome c content was high in the control and scrambled RNA-transfected cell lines.

Disturbed mitochondrial function may also induce apoptosis in a caspase-independent manner, for example, via AIF release from the damaged mitochondria. To decide if this pathway contributes to the activation of the apoptotic machinery in TASK-3 knockdown melanoma cells, AIF-specific immunocytochemistry was performed on both WM35 and A2058 cells. The results were similar in both investigated cell lines: in control and scrambled RNA-transfected cells the AIF-specific labelling showed a diffuse cytoplasmic distribution but it spared the cell nuclei. In TASK-3 knockdown cells, on the other hand, AIF immunolabelling was present in the cell nuclei, too. Because translocation of AIF from mitochondria to the cell nucleus is an essential step of caspase-independent apoptosis, these results are in agreement with the increased apoptotic activity seen in TASK-3 knockdown melanoma cells. In conclusion, our data indicate that on reducing TASK-3 expression in malignantly transformed melanoma cells, the cells die due to apoptosis. Moreover, both the caspase-dependent and the caspase-independent intrinsic pathways participate in the apoptotic cell destruction.

Investigation of the role of TASK-3 channels in melanoma cells led to the conclusion that several intracellular processes can influence the viability and the lifespan of the cells. In an earlier phase of the present work, we have examined the roles of regulatory mechanisms that were not studied in the frame of the gene silencing experiments. One of these regulatory pathways is the intracellular calcium concentration changes; another one is the neuronal/humoral muscarinergic influence. Investigation of these pathways is reasonable because the importance of both the muscarinic receptors and the calcium homeostasis has already been described in other tumour cell types. In the course of the experiments, we combined the investigation of the two related questions as cytoplasmic calcium concentration changes were evoked via muscarinic receptors (MRs) in three melanoma cell lines. The quantitative description of the MR expression was done by Western blot experiments. The presence of both receptor proteins (MR1, MR3) was clearly shown in all three melanoma cell lines studied (WM35, HT199, HT168-M1).

In order to study the functionality of the MRs, carbamyl-choline (CCh, an established cholinergic agonist) was applied, and the evoked changes in the cytoplasmic Ca^{2+} concentration were recorded. To establish the concentration of CCh suitable to elicit maximal responses on the individual cells, dose-response curves were determined on the individual cell lines. Considering the EC_{50} values obtained, 1 mM CCh (corresponding to a saturating concentration) was chosen to elicit the Ca^{2+} concentration changes in the subsequent experiments.

Some cells responded to the CCh challenge with a complex waveform that consisted of an early transient peak and a late plateau phase; the latter was maintained during the application of the CCh. In a different group of the cells only the early (transient) response was present without sustained component. In some other cases only the late (sustained) component seemed to be present. A small fraction of the cells exhibited oscillatory Ca^{2+} concentration changes during the CCh application. The two latter shapes were categorised as 'atypical' responses.

Functional and pharmacological experiments generally require repeated applications of the agonist. In the cases of the WM35 cells, the amplitude of the second Ca^{2+} transient was 93% of the first one whereas the amplitude of the third transient decreased to 49% (relative to the first one). In the cases of the HT199 and HT168-M1 cells, the decrease of the second Ca^{2+} transients was more pronounced (the amplitude reduced to 56 and 37% of the first one in the two cell lines, respectively), and the third transients were even more suppressed (to 27 and 19% of the first amplitudes, respectively). The phenomenon that the amplitude of the calcium transients decreased in the case of repeated CCh applications was called tachyphylaxis.

To justify the role of the MRs in mediating the CCh-induced cytoplasmic Ca^{2+} transients, atropine, the well-known general blocker of these receptors, was applied. The statistical analysis of the experiments showed that in the presence of atropine the size of the Ca^{2+} transients was 9, 3, and 12% of the amplitude of the first transient in the WM35, HT199, and HT168-M1 cells, respectively. It was obvious that in the presence of atropine the second transients were considerably smaller than expected on the basis of the data of the tachyphylaxis experiments.

The highly variable time course of the CCh-induced Ca^{2+} concentration changes suggested that several Ca^{2+} sources might be responsible for the genesis of these transients. Taking into account the information about the signalization pathways of the MR1 and MR3, the early component of the responses could be regarded as a result of Ca^{2+} release from internal sources. On the contrary, the formation of the plateau component seemed to depend crucially on the extracellular calcium ions as the reduction of the extracellular calcium concentration significantly decreased the duration of the late part of the CCh-induced calcium transients.

Discussion

In the frame of the work introduced in this thesis, we successfully generated stably TASK-3 gene silenced WM35 and transiently gene silenced A2058 cell cultures. The efficacy of the gene silencing was proven by demonstrating reduced TASK-3 specific mRNA content and decreased TASK-3 protein expression in the knockdown cultures. Further observations suggested impaired mitochondrial function of the knockdown cells. We established that the mitochondria of the TASK-3 knockdown cells were depolarized, and they responded with smaller mitochondrial depolarization to the application of CCCP than their control or scrambled shRNA-transfected counterparts. The amount of mitochondrial DNA was also reduced in the knockdown cultures.

Prominent modifications could also be noted in the morphology of the TASK-3 knockdown WM35 cells. Moreover, the knockdown cells presented reduced viability and a decreased rate of cell proliferation as well as increased number of cells committing to apoptosis, while the necrotic activity was not changed. According to the data, the apoptosis is induced by the activation of both caspase-dependent and -independent intrinsic pathways.

In calcium imaging experiments generation of the muscarinic agonist CCh-induced Ca^{2+} transients could be prevented by the application of atropine. The CCh-induced Ca^{2+} transients showed rather variable time courses. They generally possessed a relatively stable early and a more variable late component. It was also established that the genesis of the latter, sustained phase depends on the presence of extracellular Ca^{2+}. When comparing the data obtained in the various cell lines, the conclusion was drawn that there were some quantitative differences between the calcium homeostasis of cells in the primary tumour-originated and the metastatic cell lines. These differences may be (at least partially) responsible for the differences that exist in the biological behaviour and clinical features of the primary tumours and their metastases.

TASK-3 channels in malignant tumours

Our group has already implied that TASK-3 channels may be expressed in one of the mitochondrial membranes of the melanoma cells, and suggested that these mitochondrial TASK-3 channels contribute to the mitochondrial function. The results obtained with gene silencing techniques in different cell lines unequivocally demonstrated that interference with TASK-3 biosynthesis resulted in reduced number of melanoma cells, decreased proliferation rate, and modified morphology of the surviving cells. Our data also proved the significance of mitochondrial depolarization and increased apoptotic activity in eliciting the observed phenomena.

According to the literature, TASK-3 channels play a controversial role in the tumourigenesis. First, depending on the cell type expressing them, they seem to be able to either promote or prevent apoptosis. Second, while several articles reported strong expression of the TASK-3 channel protein in a number of malignantly transformed cells, no significant change was observed in the expression of TASK-3 mRNA in a recent study that investigated 20 cancer types. Third, there is evidence that the presence of TASK-3 may either promote the growth of the tumour or improve the prognosis of this malady.

One can conclude, therefore, that TASK-3 expression or overexpression of malignantly transformed cells is not a ubiquitous feature and they should be assessed on a case-to-case basis. At present, there is no general rule that could forecast the presence of TASK-3 protein in a given type of tumour. In addition, it is impossible to tell if TASK-3 expression of a tumour is a positive or negative prognostic feature, or it is irrelevant from the aspect of the malignancy of the disease.

These uncertainties have two notable consequences. On the one hand, the expectation that TASK-3 may serve as a prognostic marker must be, at least temporarily, dismissed. On the other hand, the clinical benefit of a therapy featuring TASK-3 inhibition cannot be guaranteed, even if selective TASK-3 inhibition could be achieved. Further studies are needed, therefore, to find reliable correlation between the TASK-3 expression and the behaviour of the malignantly transformed cells and/or prognosis of the tumour.

Although the data available about the significance of the TASK-3 channel in tumour cells do not show recognisable pattern, our results are consistent: WM35 and A2058 melanoma cells express TASK-3 channels, and when their TASK-3 expression is reduced, mitochondrial depolarization occurs and the cells are eventually destroyed. These data suggest that TASK-3 channels are essential for the survival of melanoma cells. Although further studies are needed to show if TASK-3 expression is an essential prerequisite of the survival of other (or all) melanoma cells, it is reasonable to propose that TASK-3 channels may become legitimate targets of future anti-melanoma treatments.

It has to be emphasized that the application of TASK-3 protein as a therapeutic target is made difficult by the fact that TASK-3 expression is not limited to malignantly transformed cells. Moreover, TASK-3 subunits could be replaced by TASK-1 protein. In fact, the contradictory results regarding the roles of TASK-3 protein in various tumour cells may be explained by supposing that their absence is compensated by other K_2P channels. It cannot be excluded either that alternative mechanisms may have importance in substituting TASK-3 function. An important implication of this consideration

is that if similar compensatory mechanisms are present in melanoma cells; i.e., the lost TASK-3 function is substituted by TASK-1 channels or by other mechanisms, a TASK-3-based therapeutic approach may prove less effective than hoped. Importantly, such a substitution did not seem present in our experiments; at least the amount of TASK-1 protein was not changed in TASK-3 knockdown cells.

Muscarinic cholinergic receptors in melanoma cells

The presence and functionality of the various odd-numbered MRs have been shown in melanoma cells using several methods and cell types. With the help of a monoclonal antibody recognizing all MRs, for example, the density of the immunopositivity was compared in human samples of surgical origin. It was found that the amount of MR proteins was greater in the periphery of the tumours, indicating a correlation between the MR expression level and the migratory or metastasis-forming capacity of the cells.

In this study we successfully showed the presence of both MR1 and MR3 protein subtypes in all three investigated cell types. In the same time we did not find significant differences in the amount of the proteins expressed by the primary (WM35) and the metastatic cells (HT168-M1, HT199).

Some data in the literature indicate that inhibition of MR3 can prevent the growth and metastasis formation of certain tumours. Although the MRs may activate a number of different downstream signalization mechanisms in melanoma cells as well, the involvement of the Ca^{2+} in this complex regulatory system has been well established. For example, a considerable number of studies indicated that, similarly to many other cell types, the proliferation and the motility of melanoma cells might be regulated through the cytoplasmic Ca^{2+}. Considering these data it seemed reasonable to analyse the properties of MR-induced calcium transients.

On studying the CCh-induced calcium transients, we found that the MR-related Ca^{2+} concentration increases in the three cell lines usually contained a rapid early phase that might reflect Ca^{2+} release from internal stores. It is unlikely, however, that this release takes place through ryanodin receptors. Alternatively, we have to suppose that the metabotropic MRs either directly activate Ca^{2+} channels in the cell membrane or they induce inositol 1, 4, 5-trisphosphate-mediated Ca^{2+} release. The former possibility does not seem to be likely based on the observation that the early component of the MR-induced Ca^{2+} transients did not depend on the extracellular Ca^{2+} concentration, whereas the second possibility is supported by the known signalization connections of the odd-numbered MRs. As for the late component of the CCh induced Ca^{2+} transients, its dependence on the

extracellular Ca^{2+} was demonstrated both in our experiments (MR1, MR3) and in the literature (MR5).

Therapeutic considerations

Despite many encouraging reports on novel, potentially successful approaches in the therapy of malignant melanoma, the overall survival rate of metastatic melanoma is disappointingly low. The difficulties related to melanoma treatment are, at least in part, associated with the fact that melanoma cells have defective pro-apoptotic signalling. Not surprisingly, therefore, correcting the apoptosis deficiency of melanoma cells is considered an attractive avenue to effective melanoma treatment.

It is an important and relevant finding of the experiments presented here that the inhibition of mitochondrial Kv1.3 channels activates the apoptotic pathway in tumour cells (including melanoma cells) and eventually kills the cells. In our experiments activation of the apoptotic machinery and the subsequent death of melanoma cells were achieved by reducing TASK-3 expression. It is worth emphasizing that there are promising efforts to produce effective and selective TASK-3 channel inhibitors, which may exert similar effects. For example, spermine has already been shown to be able to inhibit TASK-3 channels. More recently the development of a 5, 6, 7, 8-tetrahydropyrido[4, 3-d]pyrimidine-derivative was reported which appears to be a potent and selective antagonist of TASK-3 channels. Application of these or similar TASK-3 inhibitors may also be considered when designing new, TASK-3-targeted and apoptosis inducing therapeutic strategies.

Possible further experiments

To ensure future practical application of the present data, it is vital to interpret them in the frame of a model. Using such model, we can plan further experiments partly to confirm, partly to fine-tune the information incorporated into the model. Based on the experimental data it seems unambiguous that the expression of TASK-3 is increased in cultured melanoma cells and the channel protein is mostly localised in the mitochondria. The results supported the conclusion that TASK-3 channels contribute to the survival of tumour cells.

There is a possibility that interaction between TASK-3 and BAX makes the TASK-3 channels impermeable, which causes mitochondrial hyperpolarisation. To confirm this idea we need to conduct experiments exploring the presence and interactions of the relevant proteins.

The mitochondrial hyperpolarisation, along with other molecules, increases the mitochondrial uptake of calcium ions. This ion can enhance the activity of some enzymes of the citric cycle and increase the production rate of ATP and ROS. This idea indicates the necessity of investigating the

metabolic activity of melanoma cells to find out the role TASK-3 protein in modifying this process.

The results presented in this thesis assume that through modifying the calcium homeostasis or other signal transduction pathways, MR can influence the viability, tumour progression, and metastasis formation of melanomas. Other results in the literature point toward the role of MRs in tumour growth, metastasis formation, and tumour vascularisation in other types of tumours. It seems to be reasonable to investigate if the same or similar effects can be identified in melanoma cells. It is highly likely that these mechanisms are also present and functional in melanoma cells.

Summary

Recent epidemiology studies indicate that the incidence of melanoma increases at an alarmingly high rate. Unfortunately, metastatic melanoma has particularly poor prognosis because it is resistant to both chemotherapy and antitumour immune response. In the present work we have studied the significance of the TASK-3 potassium channels and muscarinic cholinergic receptors in the survival and intracellular Ca^{2+} homeostasis of cultured melanoma cells.

TASK-3 channels are thought to promote proliferation and/or survival of malignantly transformed cells, most likely by increasing their hypoxia tolerance. Based on our previous results that suggested mitochondrial expression of TASK-3 channels, we hypothesized that TASK-3 channels have roles in maintaining mitochondrial activity. In the present work we have studied the effects of reduced TASK-3 expression on the mitochondrial function and survival of WM35 and A2058 melanoma cells. TASK-3 knockdown cells had depolarized mitochondrial membrane potential and contained a reduced amount of mitochondrial DNA. Compared to their scrambled shRNA-transfected counterparts, they demonstrated diminished responsiveness to the application of an established mitochondrial uncoupler. These observations indicate impaired mitochondrial function. Further, TASK-3 knockdown cells presented reduced viability, decreased total DNA content, altered cell morphology, and reduced surface area. As opposed to control melanoma cell lines, which did not present noteworthy apoptotic activity, the rate of apoptosis was about 50% in the TASK-3 knockdown cultures. Sequestration of cytochrome *c* from the mitochondria to the cytosol, increased caspase 3 activity, and translocation of the apoptosis-inducing factor from mitochondria to cell nuclei were also demonstrated in TASK-3 knockdown cells. We conclude that interference with TASK-3 channel expression induces caspase-dependent and caspase-independent apoptosis of melanoma cells, most likely via causing mitochondrial depolarization. Based on our results we submit that TASK-3 channels may be legitimate targets of future melanoma therapies.

In the second part of the work, experiments were carried out to describe differences between cultured primary and metastatic melanoma cell lines in their muscarinic acetylcholine receptor- (MR-) mediated intracellular Ca^{2+} signalization. We confirmed the expression of type 1 and type 3 MRs (MR1, MR3). The functionality of these MRs was tested by applying 1 mM carbamyl-choline (CCh) and recording the associated increases of the cytoplasmic $[Ca^{2+}]$. We found that the expression levels of the receptor proteins were not significantly different in the metastatic (HT199, HT168-M1) and the primary (WM35) cell lines. Although CCh application induced Ca^{2+} transients in all three investigated cell lines, the proportion of

responding cells was the smallest in the WM35 cell culture. The CCh-induced Ca^{2+} transients could be effectively blocked by atropine (0.1 mM). The time courses of the Ca^{2+} transients were highly variable, and in some instances they showed a late (plateau-like) component, whose presence crucially depended on the influx of extracellular Ca^{2+}. When the extracellular Ca^{2+} concentration was reduced, the duration of the CCh-evoked transients was considerably decreased. This phenomenon was more pronounced in the metastatic cell lines. We conclude that although there are no fundamental differences in the MR-mediated Ca^{2+} signalization of the primary and metastatic cell lines, the quantitative differences revealed in the present work may help to understand why metastatic cells have increased malignancy and more pronounced migratory potential than those isolated from a primary tumour.

Keywords

apoptosis, melanoma, mitochondria, muscarinic receptors, TASK-3, tumorgenesis, cytoplasmic calcium

Register number: DEENKÉTK/415/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Dénes Nagy
Neptun ID: LFU9JX
Doctoral School: Doctoral School of Molecular Medicine
Mtmt ID: 10036794

List of publications related to the dissertation

1. **Nagy, D.**, Gönczi, M., Dienes, B., Szöőr, Á., Fodor, J., Nagy, Z., Tóth, A., Fodor, T., Bai, P., Szűcs, G., Rusznák, Z., Csernoch, L.: Silencing the KCNK9 potassium channel (TASK-3) gene disturbs mitochondrial function, causes mitochondrial depolarization, and induces apoptosis of human melanoma cells.
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DOI: http://dx.doi.org/10.1097/CMR.0b013e3283414477
IF:2.187

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3. Szentandrássy, N., **Nagy, D.**, Hegyi, B., Magyar, J., Bányász, T., Nánási, P.P.: Class IV antiarrhythmic agents: New compounds using an old strategy.
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   DOI: http://dx.doi.org/10.1369/jhc.2008.950303
   IF:2.823

Total IF of journals (all publications): 17,614
Total IF of journals (publications related to the dissertation): 4,457

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

09 December, 2014