VILNIUS UNIVERSITY
INSTITUTE OF BIOCHEMISTRY

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IDENTIFICATION OF SIGNALING MOLECULES AND PATHWAYS RESPOSIBLE FOR MYOGENIC CELL SURVIVAL AFTER CARDIOTOXIC TREATMENTS

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Physical sciences, biochemistry (04 P)

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The thesis is available at the libraries of Institute of Biochemistry and Vilnius University.

VILNIAUS UNIVERSITETAS
BIOCHEMIJOS INSTITUTAS

Daiva Baltriukienė

SIGNALINIŲ MOLEKULIŲ IR KELIŲ, APSPRENDŽIANČIŲ MIOGENINIŲ LĄSTELIŲ IŠGYVENIMĄ PO KARDIOTOKSINIŲ POVEIKIŲ, IDENTIFIKAVIMAS

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Introduction

In contrast to injured mammalian skeletal muscle, which regenerates injured muscle tissue through activation of quiescent myogenic precursor or multipotent adult stem cell populations, the heart does not appear to retain equivalent reserve cell populations to promote myofiber repair. The inability of adult cardiomyocytes to divide to a significant extent and regenerate the myocardium after injury leads to permanent deficits in the number of functional cells that can contribute to the development and progression of heart failure. Consequently, the transplantation of skeletal myoblasts or various origin stem cells or cardiomyocytes derived from them into the injured myocardium is a novel and promising approach in the treatment of cardiac diseases and the restoration of myocardial function. Until now, various cell types, skeletal myoblasts among them, have been used in preclinical or clinical trials of cardiomyoplasty. Each cell type has its own advantages and limitations. Outcome of myoblasts transfer could most likely be improved by selecting muscle derived progenitors less lineage committed than myoblasts (Menasche, 2007). We propose to use the autologous skeletal muscle–derived cell lines as an instrument for repairing the damaged myocardium.

It is known that the majority (>80 %) of cells grafted into myocardium are killed within 24 h and that acute inflammation and oxidative stress play an important role in donor cell death (Suzuki et al., 2004). This may result in severe damage both to implanted cells and neighbouring cardiomyocytes. Moreover, transplanted cells have to survive in the heart for many years, and therefore they may become the targets for many cardiotoxic drugs. Thus, resistance to cell death (apoptosis or necrosis) inducing factors is of crucial importance for grafted myogenic cell survival.

Apoptosis is a widely studied phenomenon in health and disease. However, scientists have only started to clarify the role of apoptosis in the cardiovascular system and explore the therapeutic potential associated with its inhibition. It is necessary to identify the apoptotic regulatory pathways that are specific for myogenic cells. Cell death/antideath pathways are potential targets for treatment in improving cardiomyoplasty. The role of mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways are widely studied in cell apoptosis induced by various kinds of stresses. The three subfamilies of MAPKs have been identified: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 – MAPKs. It has been shown that ERKs are essential for the cell survival, while JNKs and p38 MAPKs are stress responsive kinases and thus involved in apoptosis induction. However, the regulation of apoptosis by MAPKs is more complicated and often controversial, depending on the stimuli, cell type, magnitude and duration of the MAPK activity. The members of MAPK family direct distinct cellular activities even though they share a number of common substrates, including several transcriptional factors. One of them is c-Jun, a member of inducible transcriptional complex AP-1, composed of c-Fos and c-Jun proteins, which is crucial for cell adaptation to environmental changes. The role of c-Jun in the regulation of cell fate is complicated. A central role for c-Jun regulation in the response to stress is assumed for JNK although the recent data suggest a more significant role of other kinases. There is evidence about involvement of ERK and p38 MAP kinases in the regulation of c-Jun expression and activity.

In many systems activation of the phosphatidylinositol 3-kinase (PI3K) and its downstream effector, serine-threonine kinase Akt (or protein kinase B, PKB) provide

stimulus for cell proliferation, growth, and survival. Akt functions to promote cellular survival in the heart *in vivo*. The activation of this pathway may be useful in promoting transplanted cell survival in the diseased heart.

The aim of this work was to identify signaling molecules and pathways governing the muscle-derived stem cell death or survival after cardiotoxic treatments.

The tasks of this work were as follows:

- to establish and characterize the myogenic stem cell lines from the muscle of adult organism;
- to assess apoptotic response of myogenic stem cells after exposure to cardiotoxic/genotoxic agents;
- to determine the activation characteristics of MAPKs and PI3K/Akt kinases after cardiotoxic treatment;
- to ascertain the role of individual MAPKs and Akt kinase in myogenic cell survival/death regulation.

In this study there were established the first myogenic stem cell lines derived from adult rabbit skeletal muscle. We proved their myogenic origin by detection of desmin. The data obtained revealed the ability of such cells to be maintained in tissue culture for many months without a decline in proliferation and changes in myogenesis. Their long-term proliferative capacity in culture makes this system convenient for *ex vivo* gene transfer. Genetically engineered myogenic cells with improved survival properties would be a mean to improve transplanted cells survival rates in the damaged heart tissues. There were performed primary trials of cell transplantation into rabbit heart damaged by infarct. The obtained results confirmed their suitability for cardiomyoplasty.

Cell death/anti-death pathways may be the potential therapeutic targets for the treatment and improvement of cardiomyoplasty. This work was devoted to elucidation of molecular mechanisms governing the adult muscle-derived stem cell death/survival. For the first time there was assessed the sensitivity of myogenic stem cells derived from the rabbit muscle to potential toxicity in the damaged heart. The results of the apoptotic studies, during which cultured myogenic cells were exposed to various cardiotoxic agents, proved the apoptotic mode of cell death. Patophysiological (NO donor NOC-18 and hydrogen peroxide) and genotoxic (doxorubicin and cisplatin) agents were used as apoptotic inducers.

The role of mitogen-activated protein kinases (MAPKs) and Akt kinase in the myogenic cell apoptosis regulation was evaluated. The data obtained revealed an antiapoptotic action of ERK and p38 MAP kinases. JNK signaling pathways played distinct roles in apoptosis of myogenic cells mediated by different genotoxic agents. On the other hand, the ERK, JNK, and p38 MAP kinases are implicated in the proliferation of these cells. Our results revealed that overexpression of c-Jun, the main substrate of JNK, sensitizes myogenic cells to cardiotoxic agents.

The data proved the protective role of PI3K/Akt kinase after genotoxic/cardiotoxic treatment. A potential proapoptotic role of PI3K/Akt signaling pathway was unexpectedly revealed after treatment of myogenic cells with DNA damaging drug cisplatin.

In conclusion, our data indicate that positive modulation of the activity of MAPKs and Akt during cell transplantation procedure would be a useful strategy in increasing the survival of transplanted cells.

Dissertation contents. The dissertation is written in Lithuanian and contains the following parts: Introduction, Literature review, Materials and methods, Results and Discussion, Conclusions, List of references (357 positions), List of publications, Figures (33). Total 133 pages.

Materials and Methods

Reagents. TNF-α, IL-6, NOC-18, hydrogen peroxide, cisplatin, doxorubicin, acridine orange, ethidium bromide, trypan blue and propidium iodide were purchased from Sigma-Aldrich. Inhibitors UO126, SP600125, Akt inhibitor VIII, Ly294002, wortmanin, SB203580 were from Promega or Calbiochem. RNase A was obtained from Fermentas.

Antibodies. For the assessment of activation of individual signaling molecules antibodies against ERK, phospho-ERK, JNK, HA (Santa Cruz), c-Jun, Sca-1, FasL (BD Biosciences), phospho-JNK, phospho-p38; p38, Akt, phospho-c-Jun, caspase-3 (Cell signaling Technologies), phospho-Akt (Ser473 and Thr308), m-cadherin (BIOSOURCE, Invitrogen), myosin heavy chain (MHC), desmin (Sigma), and MyoD (Abcam) were used. Anti-rabbit and anti-mouse antibodies conjugated with alkaline peroxidase purchased from Cell Signaling Technologies. Secondary antibodies Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-muose were obtained from Molecular Probes.

Isolation and cultivation of muscle-derived cells. Primary myogenic cells were isolated from white California rabbits weighing 2.0 - 2.5 kg. Animals received human care according to the "Law on care, welfare and use of animals" of Lithuanian Republic. Licence for the use of laboratory animals in stem cell research (No. 0121, 2004-07-09 and No. 0171, 2007-10-31) was received from Lithuanian Food and Veterinary office. A longitudinal incision was made intramuscularly under sterile conditions and general rabbit anaesthesia (50 mg/kg of ketamin and 5 mg/kg diazepam). A pierce of skeletal muscle tissue (0.3 cm^3) was placed on the plate with cold Hank's salt solution (Sigma), minced with scissors and exposed to digestive solution containing 0.125% trypsin-EDTA (Biological Industries), 1 mg/mL collagenase type V and 0.3 mg/mL hyaluronidase (Sigma) in phosphate buffer (PBS) as described in (Širmenis et al., 1999). After incubation for 15 min at 37° C in a shaker bath the cell mass was washed with Iscove's modified Dulbeco medium (IMDM) (Sigma), supplemented with 10 % fetal calf serum (FCS) (Biological Industries). Cells were grown in multiple polystyrene tissue culture plates, growth medium IMDM, supplemented with FCS (10 %), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were maintained at 37° C in humidified atmosphere air with 5 % CO_2 and passaged twice a week detaching cells from the plate by 0.25 % trypsin/EDTA solution.

Cell lines used for comparative analysis. For this purpose human pre-T lymphocyte Jurkat, pre-B lymphocyte REH, promyelocytic leukemia HL60, chronic myelogenous leukemia K562, promyelocytic leukemia NB4, epithelial carcinoma A431 and lung carcinoma A549 cell lines were obtained from the American tissue culture collection (ATCC). Jurkat, REH, HL60, K562, NB4 cells were cultured in RPMI-1640 supplemented with FCS (10 %), penicillin (100 U/mL) and streptomycin (100 μg/mL). Adherent cell lines – A431 and A549 were grown in IMDM with analogous concentrations of FCS and antibiotics. Cells were maintained at 37° C in humidified atmosphere air with 5 % CO_2 and passaged twice a week detaching adherent cells from the plate by 0.25 % trypsin/EDTA solution.

Viability assessment using trypan blue exclusion test. The number of viable cells was determined with a trypan blue dye (0.4% trypan blue in PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl)) exclusion test. Adherent cells were trypsinised and resuspended in growth medium. Cell suspension was mixed with trypan blue dye solution 1:1 and viability was evaluated under the light microscope. The method is based on the penetration of dye into nonviable cells colouring them blue.

Proliferation Assay. Proliferation of cells was assessed by MTT assay (Sigma). After appropriate treatments the growth medium was removed and MTT (0.2 mg/mL) solution in PBS was added (200 μL for each well of 24-well plate). After incubation at 37 ^{o}C for 1 h, the medium was replaced with ethanol (500 μL/well) and after 10 min incubation the plate was read on the automated microplate reader with a wavelength of 570 nm.

Evaluation of cell growth intensity. After appropriate treatments the growth medium was removed and cells were fixed by 0,1% crystal violet solution in 20% ethanol (200 μL for each well of 24-well plate) for 30 min. Then cells were washed with water. Before measurement dye was solubilised with 0,1% acetic acid solution in 50% ethanol. Optical density proportional to cell number was measured on the automated microplate reader with a wavelength of 570 nm.

Immunocytochemistry. Cells were grown in 30 mm diameter Petri plates with glass slides on the bottom (or in tissue culture flasks in case of flow cytometry), fixed with ice cold acetone:methanol solution (1:1) for 10 min. or 4% paraformaldehyde for 15 min. Then slides or cells were washed with PBS and incubated with 0.2% Triton X-100 in PBS for 15 minutes to permeabilize the membranes. After blocking with 1 % BSA in PBS, slides or cells were incubated with primary antibodies against desmin, MyoD or MHC for 1h at 4 ^{o}C. Then rinsed with 1 % BSA in PBS and incubated with secondary antibodies Alexa Fluor594 or Alexa Fluor488. (In case of MHC this step of incubation was not required, because the primary antibodies were conjugated with alkaline phosphatase. After incubation with primary antibodies cells were visualized by light microscope). Then analysis by confocal microscope or flow cytometry was performed.

Induction of differentiation. Cells were cultured in differentiation medium (DM; DMEM supplemented with 3 % horse serum) for 3 days and evaluated by cell fusion as well as expression of MHC. Cell fusion was visualised by standard light microscope using 0,1% crystal violet dye.

Graft cell labelling. Prior the transplantation, cultured myogenic cells were grown with DAPI (10 μg/ml) for 2 hours. Then the labelled stem cells were detached from the plate by 0.25 % trypsin/EDTA solution, spun down, washed with PBS and resuspended in serum-free growth medium (5 x 10^6 cells/mL) for grafting.

Histological analysis. At the end of the experiment the rabbits were killed in a humane way according to the Animal welfare Laws and their hearts were excised. The histological analysis of heart sections was performed in two ways: the first series was stained with hematoxylin/eosin and examined under the standard light microscope, the second series was analysed by fluorescent microscope and it showed engrafted DAPI-labelled cells. Both analyses were done in one month after the transplantation of myogenic cells.

Cell cycle analysis. Cells were treated with apoptotic inductors for 24 h and fixed in cold 70 % ethanol for 24 h at -20 ^{o}C. Before the analysis cells were washed with PBS. After centrifugation at 500 × g for 5 min, the cells were suspended in PBS containing propidium iodide (PI) (50 μg/mL) and RNase A (0.1 mg/mL) and incubated at room temperature for 30 min. The tubes were then kept at 4 °C in the dark until analysis by flow cytometry (ex 505, em 615) (BD FACSCanto II, USA). The percentage of cells in G0/G1, S and G2/M was evaluated with BD FACSDiva software. Apoptotic cells were quantified on a PI histogram as a hypodiploid peak, and the data were registered on a logarithmic scale.

Apoptosis assay. Apoptotic index was determined using two fluorescent dyes: acridine orange (AO) and ethidium bromide (EB). AO was used to characterize chromatin condensation and EB - to characterize the membrane integrity. Cells were categorized as follows: V - viable with non-fragmented nuclei (bright green chromatin); VA - viable with fragmented nuclei (bright green chromatin with organized structure); N – necrotic (red nonfragmented nuclei); NA - nonviable apoptotic with fragmented nuclei (bright orange chromatin that is highly condensed or fragmented). Chromatin-free cells lost their DNA content entirely and exhibited weak green-orange staining (Mercille & Massie, 1994).

DNA fragmentation assay. $1x10^6$ cells were treated with 50 µL of lysis buffer (50 mM Tris-HCl pH 7.5, 1 % NP-40, 20 mM EDTA). After centrifugation for 5 min at 1600 x g, the supernatant was collected and the extraction was repeated with the same amount of lysis buffer. The supernatant was treated for 2 h with RNase A (5 mg/mL) at 56 ^oC followed by digestion with protease K (2.5 mg/mL) for 2 h at 37 ^oC. 1/2 volume of 10 mM ammonium acetate was added and DNA was precipitated with 2.5 volume of ethanol, dissolved in gel loading buffer and separated by electrophoresis in 2 % agarose gel. DNA was then visualized under UV light after gel staining with ethidium bromide.

Transfection. One day before transfection myogenic cells were plated at the density 5 x 10^4 cells/cm^2 in IMDM with 10 % FCS and without antibiotics. On the day of transfection DNA (the sequence of *c-jun* gene cloned into a plasmid pcDNA3 (Invitrogen)) and LIPOFECTAMINE^{TM} 2000 (Invitrogen) were diluted separately in OPTI-MEM (Invitrogen) without serum according to the manufacturer's recomendations. After 5 min of incubation, DNA was combined with diluted LIPOFECTAMINE^{TM} 2000, mixed gently and incubated for 40 min at room temperature. Total volume of mixture was added to the target cells and incubated for 6 h at 37^oC. After incubation the medium was replaced by growth medium containing 20 % FCS. Three days later the selective antibiotic geneticin (final concentration 50-200 µg/mL) was added.

The expression construct for hemaglutinin-tagged version of constitutively active form of Akt in pBabe-puro vector (von Gise et al., 2001) was used for modulation of Akt kinase expression.

Electroporation. Cells were trypsinised and counted. $5x10^5$ cells were spun down and washed once in Opti-MEM before resuspension in 200 µL Opti-MEM. Cells were then incubated with 600 nM siRNA for 10 min. at room temperature. Cells were electroporated using Gene Pulser XCell (Biorad) at a setting of 950 µF and 200 V in a 0.2-mm gap cuvette. Following electroporation, cells were incubated at room temperature for 20 min. After that IMDM containing 20 % FBS was added to the cells, and they were plated at a density of 5 $x10^4$.

Infection of target cells. On the day before infection, virus producing PA317 cells were plated such that they would be roughly 50-80 % confluent at the time of infection. Next day the growth medium of PA317 cells was changed into DMEM containing 3 % FBS. After 8-12 h of incubation, medium was collected, mixed with 4µg/mL polybrene, and added to the myogenic cells. After 12-24 h myogenic cells were treated with selective antibiotic geniticin (final concentration 50-200 µg/mL). Control or not transduced cells died at this concentration of antibiotic.

Retroviral vector pLNCX with the *c-jun* protooncogene sequence in antisence orientation under control of cytomegalus virus (CMV) promoter was constructed by dr. Algirdas Žiogas in our laboratory.

Immunoblotting. Cells were lysed in ice-cold lysis buffer (in mM: 10 TrisHCl pH 7.4, 50 NaCl, 5 EDTA, 50 NaF, 2 Na3VO4 with 0.1% BSA, 1% Triton X-100 and supplemented with protease inhibitors: aprotinin (20 µg/mL) and PMSF (1 mM)). Cells that became nonadherent after the treatment were combined with those that remained adherent. Equal amounts of protein were separated by SDS-PAGE on 10% acrylamide gels. Protein concentrations were estimated by the Bradford assay (Fermentas). Separated from the gel

proteins were transferred to PVDF membrane (ROTH, Germany) and the blots were blocked by blocking buffer: 5% low fat milk in TBST (4 mM Tris HCl, 4 mM Tris base saline, 0,1% Tween 20, 154 mM NaCl) for 1h at room temperature (RT). The membranes were washed in TBST three times (5 min per wash), probed first with the primary antibody in 5% low fat milkin TBST for 24 h at 4 ºC, and then with the secondary antibody in the same 5% low fat milk in TBST for 1h at RT. After the incubation, membranes were washed 3 x 5 min. in TBST. Proteins then were visualized by the Amersham ECL system.

Statistical analysis. Data are expressed as the representative result or as mean of at least three independent experiments ± SD. Statistical analyses were performed using Student *t* test. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Characterization of skeletal muscle derived cells. We have established numerous myogenic cell lines from rabbit muscle (Fig. 1). A muscle-specific phenotype of these cells was confirmed by the expression of desmin, a marker of myogenic cell origin. Early and late passage cultivated cells were equally identified with antibody against desmin, which was expressed in both proliferating as well as in differentiating cells, cultivated in DM for 3 days. Cells from different lines were capable to differentiate into multinucleated, myosin heavy chain

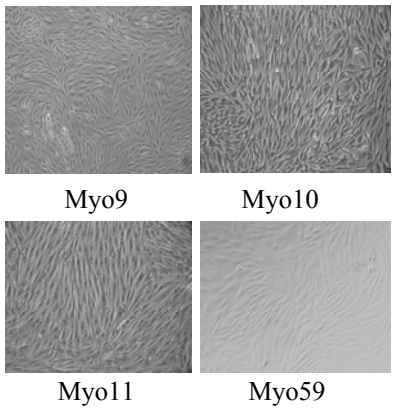


Fig. 1 Separate myogenic cell lines growing in monolayer. Cell lines (No 9, 10, 11, and 59) were established from skeletal muscles of different rabbits (see Materials and Methods for details).

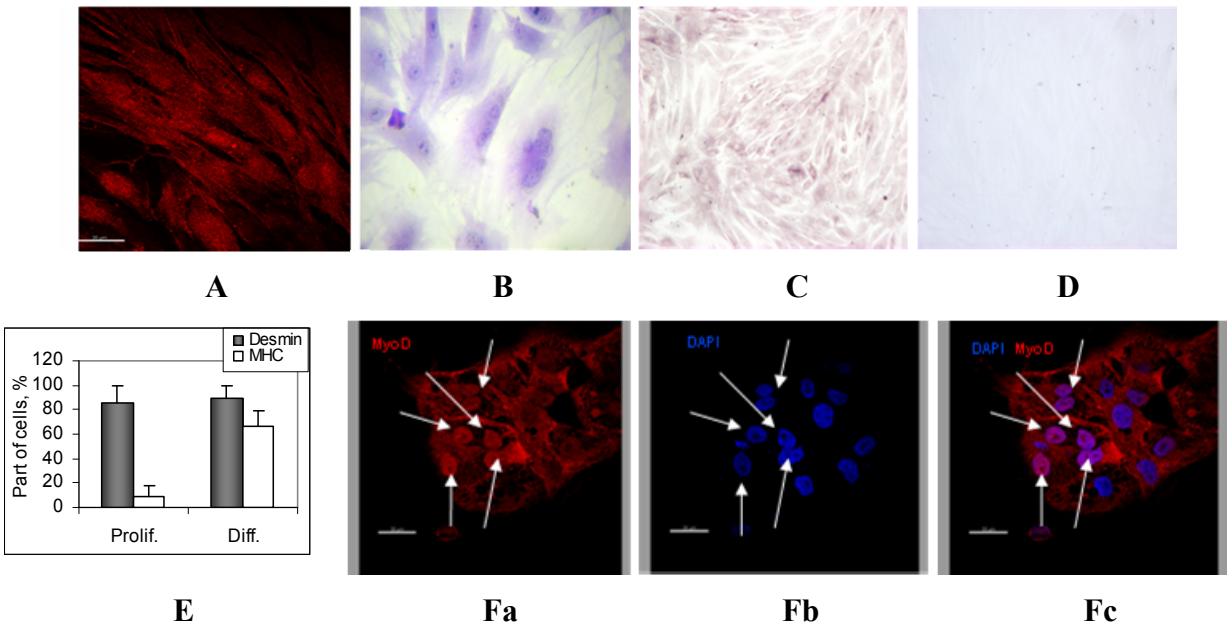
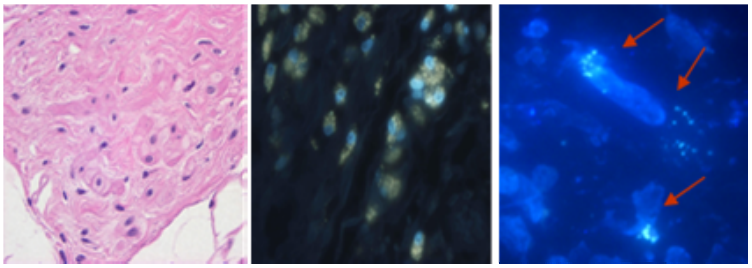


Fig. 2. Characterization of myogenic cells according to specific markers. Skeletal muscle-derived stem cells express desmin (A) and are capable to differentiate into multinucleated (B) myosin heavy chain (MHC) expressing cells (C). (D) – Control, MHC negative, cells. (E) - Differences of desmin and MHC expression in proliferating and differentiated cells, according to flow cytometry data. (F) - MyoD expressing cells – anti-MyoD (a, arrows) and DAPI (b) stained cells, complex image (c).

(MHC) expressing cells (Fig. 2, A-E). MHC usually marks the late stages of myogenic differentiation (Iezzi et al.,2002; Zammit et al., 2006). Moreover, muscle-derived cell lines express transcription factor MyoD, a marker of activated satellite cells (Yablonka-Reuveni et al., 2008) (Fig. 2, Fa-Fc). One of the key characteristics of stem cells is their capacity to divide for long periods of time in the environment where most of the cells are quiescent. We did show that myogenic cells from rabbit muscle could be maintained in tissue culture for many months without the declining in their proliferation potential (Bukelskienė et al., 2005). The possibility of prolonged cultivation of myogenic cells *in vitro* and the ability to differentiate are the features of stem cells. It extends their therapeutic potential and makes the use of our cell system convenient for *ex vivo* gene transfer. Genetically engineered myogenic cells with improved survival properties would be a mean to solve the problem of poor survival rate of transplanted cells in damaged heart tissues.

The primary trials of cell transplantation into rabbit heart, damaged by the artificial infarct, were performed. Our data indicates the integration of muscle derived stem cells into rabbit heart confirmed one month after transplantation (Fig. 3) (Bukelskienė et al., 2005; Širmenis et al., 2007). Therefore, due to their innate ability to propagate in large numbers and naturalize *in vivo* skeletal muscle-derived stem cells are becoming a potential choice for the cardiac repair.



A **B** **C**

Fig. 3. Grafted myogenic cells one-month after transplantation into rabbit heart. (A) - Light microscope image of heart section with grafted cells stained with hematoxylin and eosin. (B) - Fluorescence microscopy image of heart section with grafted cells stained with DAPI 2 h before the transplantation. (C) – Apoptotic cells in the heart section stained with DAPI one week after transplantation.

The potential of cardiotoxic agents to induce apoptosis in myogenic cells. The major limiting factors in cell transplantation are an extensive loss of cells due to early cell death and poor survival of cell graft. It was shown that over 80 % of grafted myoblasts die during the first days after transplantation. The poor survival rate is attributed to the release of the free radicals and to the inflammatory reactions of the infarcted myocardium (Beauchamp et al., 1999; Irintchev et al., 1995; Pouzet et al., 2000; Suzuki et al., 2004). The transplanted cells must be able to survive for years in the heart and they might be the targets for various kinds of cardiotoxic drugs.

Acute myocardial infarction is accompanied by increased level of cytokines (TNF-α and IL-6). The upregulation of these cytokines was detected within the myocardium at 24 h after myoblast transplantation to the heart (Suzuki et al., 2004). Therefore, our primary attention was focused on such cytokines as apoptotic inducers. However, the time course data did not reveal the cytotoxic effect on skeletal myogenic cells after exposure to TNF-α and IL-6. Moreover, there was registered a slight stimulation of cell proliferation (Fig. 4).

According to literature data, an initial 24 h period of myoblast proliferation following TNF-α treatment is associated with transient NF-κB and Jun kinases (JNK) 1

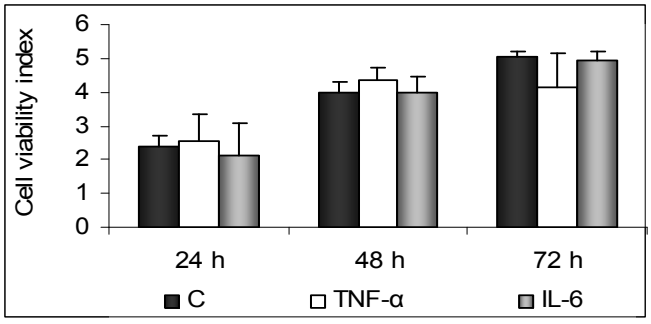
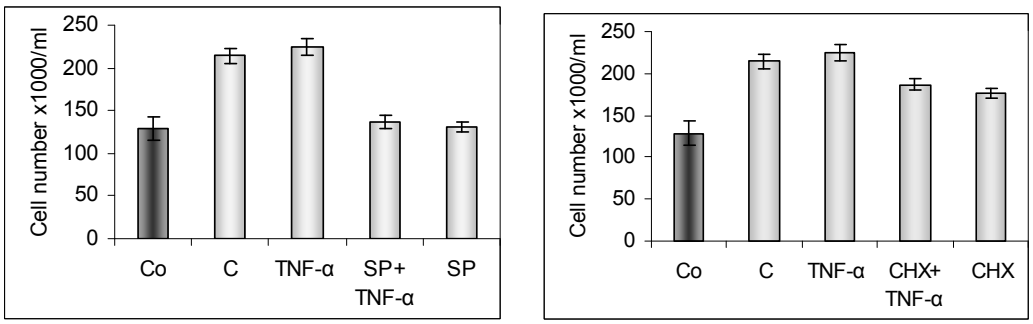


Fig. 4. Cytokines did not influence the myogenic cell viability. Myogenic cells were treated with TNF-α (150 ng/ml) and IL-6 (300 VV/ml) at the indicated time points. Cell viability index was expressed as the ratio of viable cell number after 24 hours exposure to viable cell number before treatment. Data are expressed as mean ± SD from at least three measurements.

and 2 activation followed by induction of apoptosis (Stewart at al., 2003). JNK is known to be an antiapoptotic factor in a few cells (Ogunwobi and Beales, 2007). On the other hand, cells contain apoptosis inhibitory proteins controlling the constitutively expressed apoptosis machinery. Inhibition of macromolecular synthesis in concert with TNF- α has been shown to induce apoptosis in various kinds of cells. Our data, presented in Fig. 5 show that JNK specific inhibitor SP600125 and protein synthesis inhibitor cycloheximide did not affect the TNF-α-treated myogenic cell viability.



A **B**

Fig. 5. Inhibition of c-Jun N-terminal kinase (A) and *de novo* protein synthesis (B) did not sensitize myogenic cells to TNF-α exposure. Myogenic cells were pretreated with SP600125 (SP, 40 μM) and cycloheximide (CHX, 1 μg/ml) for 30 min before addiing TNF-α (150 ng/ml). Cell number was registered after 24 h exposure by trypan blue exclusion test. (see Materials and Methods for details).

Besides, for our apoptotic studies we chose another set of apoptotic inducers: chemotherapeutic agents – cisplatin (CisPt) and doxorubicin (DOX) as well as patophysiological apoptotic inducers – NO donor NOC-18 and hydrogen peroxide (H_2O_2), all known to be cardiotoxic agents. It is also known that cell damages induced by these substances modulate signaling molecules participating in cell death/survival regulation. In our experimental model myogenic cells were exposed to cardiotoxic agents 24 h after their seeding. Various concentrations of CisPt (1-20 μg/ml), DOX (0.5-5 μM), NOC-18 (1-5 mM), and H_2O_2 (0.1-2 mM) were applied. The data obtained revealed the dose-dependent cytotoxic effect. The results of the apoptotic studies during which cultured myogenic cells were exposed to named cardiotoxic agents for 24 h proved the apoptotic mode of cell death. Apoptosis was confirmed by different methods - microscopically by the detection of cell morphological changes, cleavage of caspase 3, DNA fragmentation as well as by the cell cycle analysis (Fig. 6, A-F). The doses of drugs, required to reduce the initial cell number by 40-60 %, were used in further experiments.

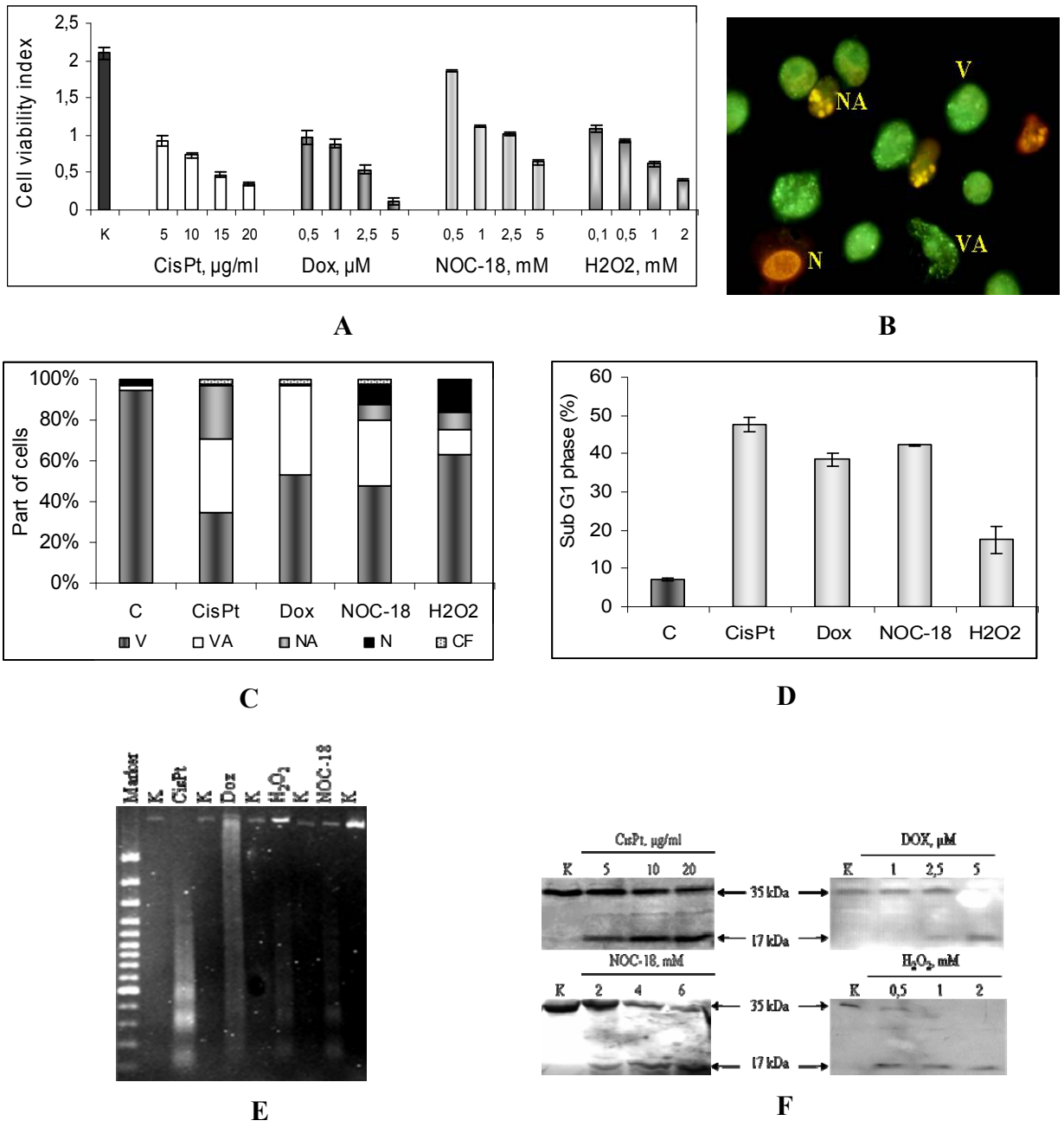
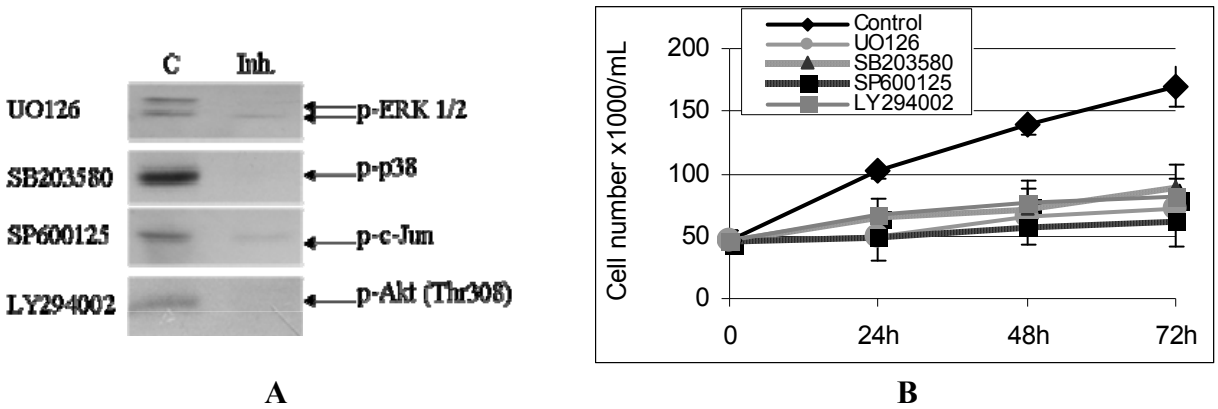


Fig. 6. Cardiotoxic agents induce apoptotic cells death in myogenic cell population. (A) – Myogenic cells were treated with increasing concentrations of CisPt, DOX, NOC-18, and H_2O_2 for 24 h. Cell viability index was expressed as ratio of viable cells after 24 hours exposure to viable cells before treatment. Data are expressed as mean ± SD from at least three measurements; $P<0.02$; (B) – Representative image of cell morphological changes induced by cisplatin. Morphological changes after exposure to other agents were analogous; (C) – Distribution of V – viable, VA – viable apoptotic, NA – nonviable apoptotic, N – necrotic, CF – chromatin free cells in myogenic cell populations after 24 h treatment with 10 µg/ml CisPt, 2.5 µM DOX; 5 mM NOC-18, and 1 mM H_2O_2. (D) – Apoptosis was proved as a hypodiploid peak (subG1) from flow cytometric analysis of PI stained myogenic cells. An additional evidence of apoptotic death was provided by DNA fragmentation (E) and caspase 3 cleavages (F).

Signaling pathways governing cell death/survival. Cellular survival or death is regulated by various signaling pathways. Recent investigations of pro-survival and apoptotic signal transduction pathways in the cardiac cell have revealed the important

role played by MAPKs (Andreka et al., 2001). The MAPK signaling pathway consists of a sequence of successively acting kinases. Three major MAP kinase cascades have been identified in myocardium – ERK1,2 and stress activated MAPKs subfamilies SAPK/JNK and p38. MAPK signaling pathways control fundamental cellular processes such as proliferation, differentiation, and cell death (Garrington and Johnson, 1999; Widmann et al., 1999). Akt (also known as protein kinase B), which is a central component of the phosphoinositide 3-kinase (PI3K) signaling pathways, has also emerged as a pivotal regulator of many cellular processes. The role of ERK MAPK and Akt kinases in cell proliferation is well known and it is associated with stimulation of positive cell cycle regulators and inhibition of antiproliferative genes. Stress activated MAP kinases JNK and p38 are generally implicated in apoptosis induction, but their role in cell proliferation has also been reported. According to literature data, JNK is activated in a cell cycle-dependent manner and plays an important role in cell proliferation with possible functions in S and G2/M phases (Du et al., 2004; Smith et al., 1997). p38 has been also implicated in G1 and G2/M phases of the cell cycle (Zarubin ir Han, 2005). Furthermore, it has been shown that this kinase is required for UV-induced G2 cell cycle arrest (Wang ir kt., 2000).

We analyzed the role of ERK, p38, JNK and Akt kinases in myogenic cell proliferation using these kinases specific inhibitors U0126, SB203580, SP600125 and LY294002, respectively. The data obtained revealed the inhibition of phosphorylation of target molecules and a marked slowdown of cell proliferation after the treatment with signaling molecules inhibitors (Fig. 7). The used inhibitors concentrations did not influence myogenic cell viability. Therefore, our data indicate that ERK, p38, JNK MAP and Akt kinases are required for myogenic cell proliferation.



A **B**

Fig. 7. MAPK and Akt kinases are implicated in myogenic cell proliferation. (A) – An inhibitory effect of U0126, SB203580, SP600125, and LY294002 on kinases was confirmed by Western blot analyzing phosphorylation of ERK1/2, p-38, c-Jun, and Akt, respectively. (B) – Myogenic cells were treated with U0126 (20 μM), SB203580 (20 μM), SP600125 (40 μM) and Ly294002 (20 μM) for indicated time points. Control cells were exposed to equal amount of DMSO (inhibitor solvent). Cell number was registered by trypan blue exclusion test as described in Materials and Methods.

As the MAP kinases have been associated with the signaling pathways responsive to apoptosis induced by various stresses we have examined whether CisPt, DOX, NOC-18, and H_2O_2 activate MAP kinases, ERK, p38 and JNK, in myogenic cells. The activation of MAPKs was investigated by Western blotting using anti-phospho-ERK, anti-phospho-JNK and anti-phospho-p38 antibodies. The membranes were also probed with antibodies against the total amount of ERK, JNK and p38.

ERK activity correlates with myogenic cell survival. ERK MAPK is known to play a crucial role in the survival of cardiomyocytes. It is important for cell division, migration, differentiation as well as for regulation of apoptosis (Gustafsson ir Gotlieb, 2003; Ravingerová et al., 2003; Werlen et al., 2003). Western blot analysis of ERK MAPK activation in myogenic cells did not reveal major changes in phosphorylation of ERK after CisPt, DOX and H_2O_2 treatments. Only NO donor NOC-18 induced late and sustained ERK activation. (Fig. 8, A). The data obtained did not reveal any changes in the expression of ERK1/2. The potential involvement of ERK in cardiotoxic treatments-induced apoptosis was studied using a selective inhibitor of MEK1/2 – U0126. MEK 1/2 is an upstream kinase of ERK and its potential activator. The obtained data indicated the protective role of ERK in CisPt-, DOX-, NOC-18- and H_2O_2–induced myogenic cell death (Fig. 8, B).

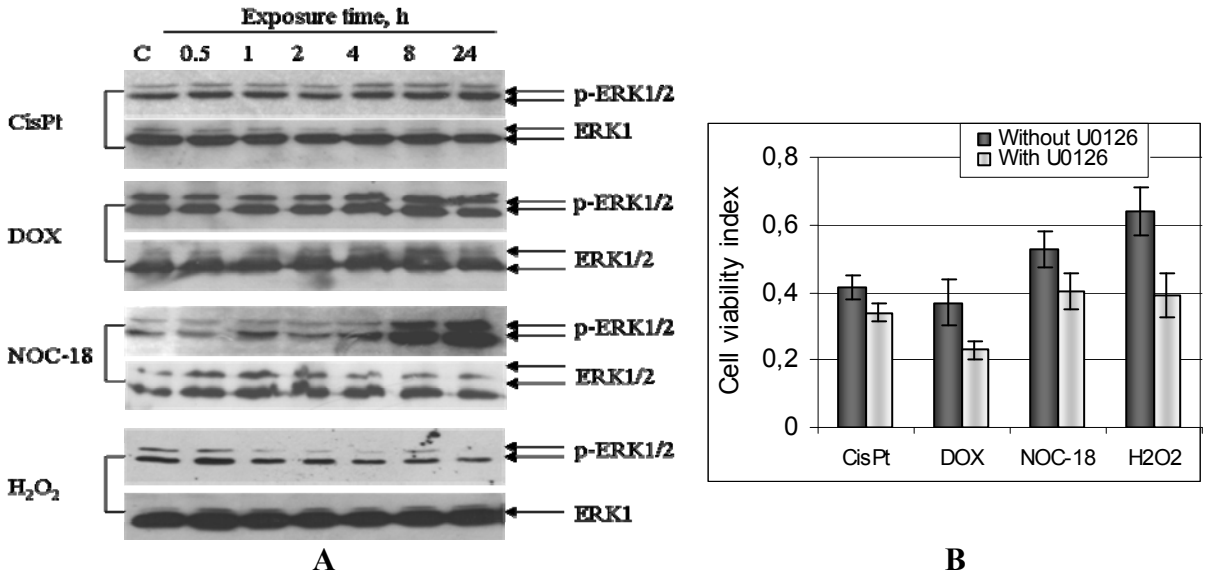


Fig. 8. The role of ERK in myogenic cell apoptosis induced by cardiotoxic treatments. (A) – Phosphorylation and expression of ERK 1/2 after exposure to cardiotoxic agents. Cells were treated with 10 μg/ml CisPt, 2.5 μM DOX; 5 mM NOC-18, and 1 mM H_2O_2 at indicated time points and analyzed by Western blotting. (B) - The effect of MEK 1/2 inhibitor U0126 on myogenic cell viability. Cells were exposed to 20 μM of U0126 for 30 min prior to adding cardiotoxic agents. Results are expressed as ratio of viable cells after 24 hours exposure to viable cells before treatment. Data are expressed as mean ± SD from at least three measurements; the significance of results with CisPt treated cells was $P<0.02$, in other cases $P<0.05$.

p38 MAPK activity correlates with myogenic cell survival. p38 MAPK has also been involved in cardiac myocyte apoptosis *in vivo* and *in vitro*. For example, ischemia and doxorubicin applied to cardiomyocytes activated p38 kinase and induced apoptosis, which was attenuated by pharmacological p38 inhibitors (Mackay and Mochly-Rosen, 2000; Sharov et al., 2003). Transgenic mice with dominant-negative MKK6 show reduced myocardial infarction suggesting a pro-apoptotic role for p38 kinase *in vivo* (Kaiser et al., 2004). On the other hand, a protective role of p38 has been shown in adult cardiomyocytes after stimulation of β- adrenergic receptors (Communal et al., 2000).

The study of p38 MAP kinase activation/phosphorylation in myogenic cells showed that this kinase is activated in a different manner depending on the apoptotic inducer used. The transient activation of p38 MAPK with a peak at 30 min was detected

in myogenic cells after H_2O_2 treatment. In contrast, slight, gradual and prolonged increase in p38 phosphorylation was registered after exposure of cells to CisPt and NOC-18. The effect of DOX on p38 phosphorylation was negligible (Fig. 9, A). No changes in the expression of p38 were found either. Pretreatment with the p38 MAPK inhibitor SB203580 increased myogenic cell sensitivity to all tested apoptosis inducers (Fig.9, B). These results indicate that p38 functions as an anti-death signaling molecule in cultured myocytes.

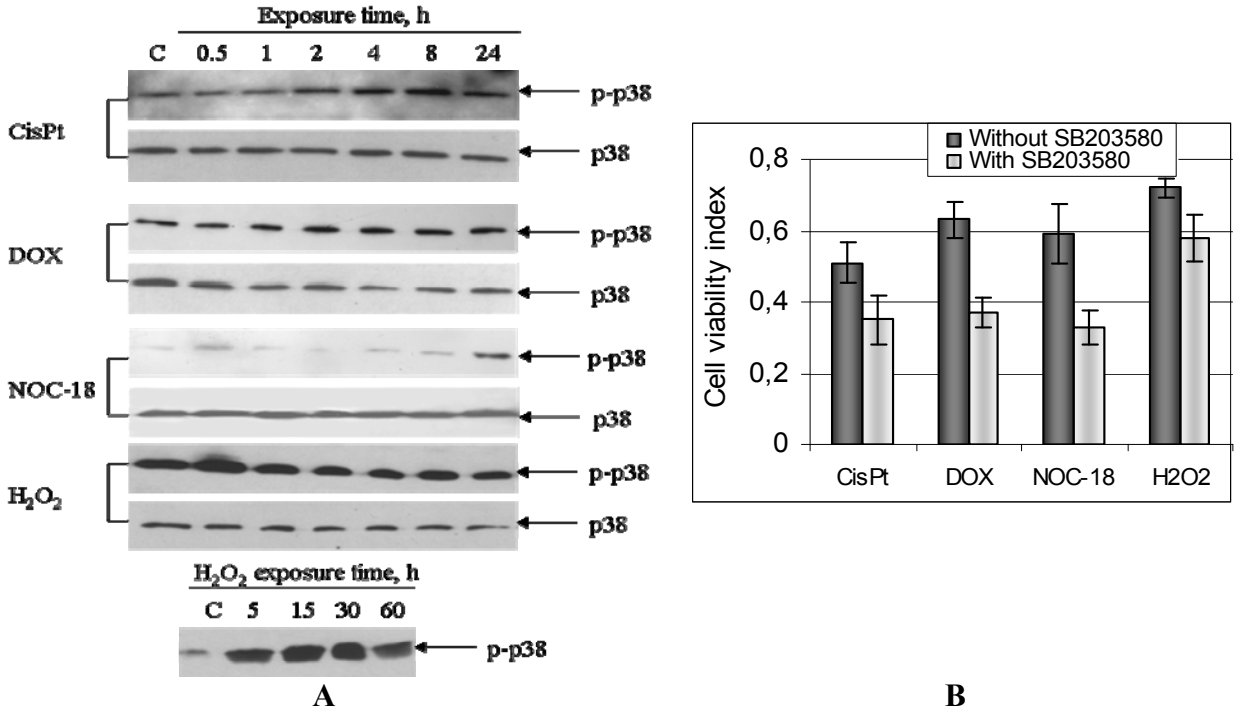


Fig. 9. The role of p38 MAPK in myogenic cell apoptosis induced by cardiotoxic treatments. (A) - Phosphorylation and expression of p38 MAPK after exposure to cardiotoxic agents. Cells were treated with 10 μg/ml CisPt, 2.5 μM DOX; 5 mM NOC-18, and 1 mM H_2O_2 at indicated time points and analyzed by Western blotting. (B) - The effect of p38 MAPK inhibitor SB203580 on myogenic cell viability. Cells were exposed to 20 μM of SB203580 for 30 min prior to adding cardiotoxic agents. Results are expressed as ratio of viable cells after 24 hours exposure to viable cells before treatment. Data are expressed as mean ± SD from at least three measurements; in all cases $P<0.05$.

Thus, the data obtained reveal, that ERK and p38 MAPK signaling pathways are involved in myogenic cell survival after cardiotoxic agents treatment. The approaches aimed at increasing the activity of these kinases in transplantable myogenic cells would be a favorable strategy in order to increase such cell survival in diseased heart.

The role of JNK in myogenic cell apoptosis. The activation of c-Jun N-terminal kinase/stress activated kinase (JNK/SAPK) pathway has been supposed to be critical for the induction of apoptosis by various stresses and mitogenic stimuli in most systems *in vitro* and *in vivo* (Johnson et al., 1996, Kim et al., 2002; Mayr et al., 2002). It has been demonstrated that β-adrenergic receptor-stimulated apoptosis of cultured adult cardiomyocytes was inhibited by dominant negative JNK (Remondino et al., 2003). JNK deficient mouse embryonic fibroblasts were resistant to UV-induced apoptosis (Tournier et al, 2000). JNK was shown to be pro-apoptotic in animal models of acute myocardial infarction (Zaheer et al, 1998). On the other hand, there is evidence that activation of JNK might also serve as a cytoprotective mechanism. The inhibition of JNK activation

results in increased apoptosis in cardiomyocytes, as well as in C2 skeletal myoblasts treated with TNF-alpha, suggesting the antiapoptotic role for JNK (Cicconi et al., 2003; Stewart et al., 2004). The activation of JNK in cardiomyocytes blocked ischemia-reperfussion-induced apoptosis (Dougherty et al., 2002).

The results of our study show CisPt- and DOX-induced sustained JNK phosphorylation (Fig. 10, A). There was observed an increase in cell number over control cell count following simultaneous treatment of chemotherapeutic drugs and JNK inhibitor SP600125. Therefore, the data obtained indicate that during CisPt and DOX treatment JNK is a proapoptotic kinase in myogenic cells and that prolonged JNK activation correlates with its proapoptotic role. Moreover, we have also observed sustained JNK activation in these cells after their exposure to NOC-18. The use of SP600125 revealed the protective role of JNK kinase in NO-induced myogenic cell death (Fig. 10, A and B). These results contradict the notion that prolonged activation of JNK is related to its proapototic role. The activation of JNK MAPK after adding H_2O_2 was transient with the peak at 30 min and return to basal level in 1-2 h. We have found that cell pretreatment with the specific JNK inhibitor SP600125 increased H_2O_2-induced apoptosis, which suggests a protective role of JNK MAP kinases in myogenic cell apoptosis induced by oxidative stress exemplified by H_2O_2**.** These results correlate with the literature data. It has been reported that early, transient JNK activation is able both to delay and reduce the apoptosis in cardiac myocytes (Andreka et al., 2001).

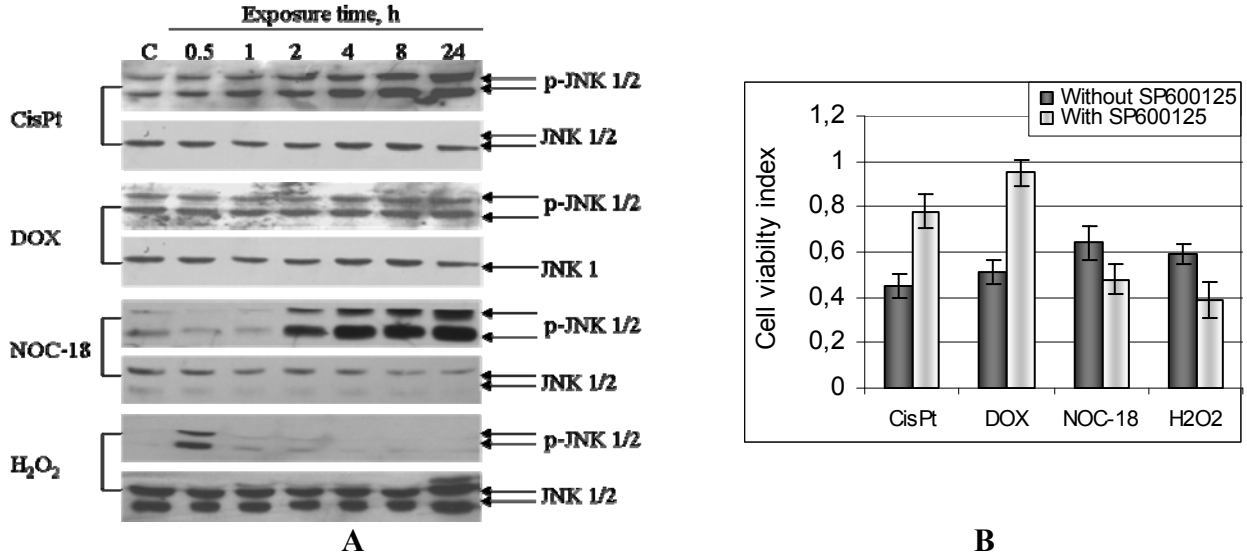
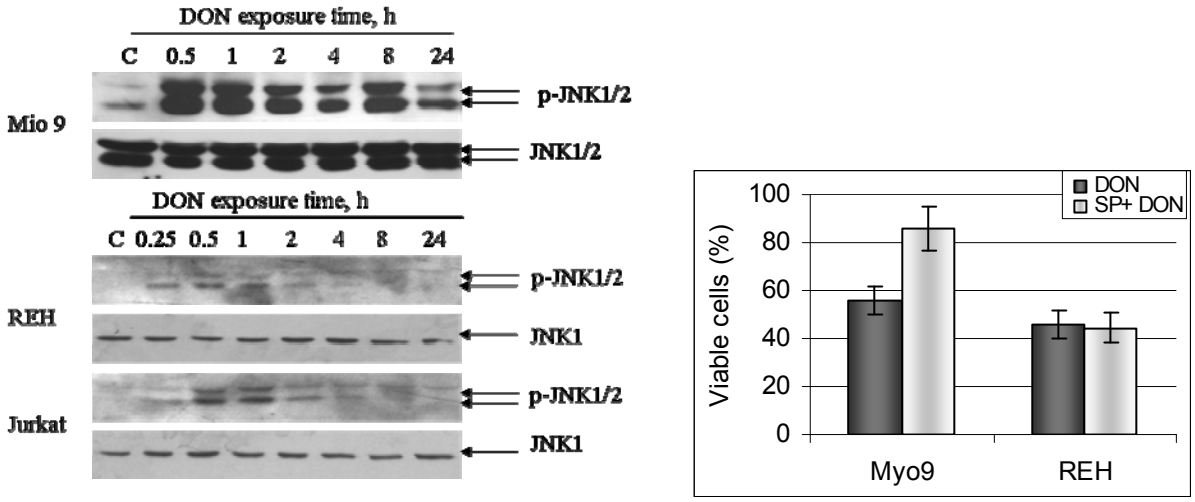


Fig. 10. The role of JNK in myogenic cell apoptosis induced by cardiotoxic treatments. (A) – Phosphorylation and expression of JNK after exposure to cardiotoxic agents. Cells were treated with 10 μg/ml CisPt, 2.5 μM DOX; 5 mM NOC-18, and 1 mM H_2O_2 at indicated time points and analyzed by Western blotting. (B) - The effect of JNK MAPK inhibitor SP600125 on myogenic cell viability. Cells were exposed to 40 μM of SP600125 for 30 min prior to adding cardiotoxic agents. Results are expressed as ratio of viable cells after 24 hours exposure to viable cells before treatment. Data are expressed as mean ± SD from at least three measurements; in all cases $P<0.05$.

Different durations of MAPKs phosphorylation are supposed to be associated with their opposing functions in cell apoptosis regulation. The persistent activation of JNK and p38 signaling pathways is known to be critical for apoptosis to occur (Mansouri et al., 2003). As discussed in the previous section, after exposure to CisPt and DOX sustained JNK activation correlated with its proapoptotic action. Similarly, the sustained activation of JNK in myogenic cells was found after NOC-18 treatment. However, in the

latter case the role of JNK was protective. In order to ascertain that the mode of JNK activation and its role in apoptosis are cell type-dependent, we chose another experimental model involving use of mycotoxin deoxynivalenol (DON). As reported earlier, DON is an effective inducer of apoptosis and MAPK activation in leukemic and other kinds of cells (Baltriukienė et al., 2007; Zhou et al., 2005). Therefore, we performed a comparative analysis of JNK activation and its role in myogenic and leukemic cells. The studies of JNK activation in leukemic Jurkat and REH cells after DON treatment revealed early and transient JNK phosphorylation. 40–60 % of cell death in both kinds of cells and prolonged JNK activation in myogenic cells were induced by the same DON concentrations (Fig. 11, A). Furthermore, JNK-specific inhibitor SP600125, protected myogenic cells from DON-induced cytotoxicity showing the proapoptotic JNK role. In contrast to myogenic cells, pretreatment with SP600125 had a negligible or negative effect on viability of REH (Fig. 11, B) and Jurkat cell (Pestka et al., 2005), respectively.



A **B**

Fig. 11. A comparative study of the role of JNK in myogenic and leukemic cells. (A) **–** Time course of phosphorylation and expression of JNK in myogenic, REH and Jurkat cells after exposure to 5 μg/mL DON was detected by Western blotting. (B) - Mio9 and REH cells were exposed to 40 μM of SP600125 for 30 min prior to adding 5 μg/mL DON. Results are expressed as percentage of viable cells after 24 hours exposure against viable cells before treatment. Data are expressed as mean ± SD from at least three measurements, the significance of results with DON treated myogenic cells was $P<0.05$.

Thus, the data obtained indicate that depending on the cell type, the same apoptotic agent may induce JNK activation of different duration. Prolonged activation of JNK in myogenic cells correlates with its implication in DON-induced apoptosis, whereas transient JNK activation in leukemic cells coincides with its prosurvival role in Jurkat cells (Pesta et al., 2005) or neutral - in REH cells (Baltriukiene et al., 2007).

The role of transcription factor c-Jun in myogenic cell apoptosis. Delineation of the sequences of signaling events leading the stem cell to death is essential for the development of strategies designed to improve their survival after transplantation. During this study we investigated possible downstream molecular targets in JNK proapoptotic signaling. c-Jun, a component of transcription factor AP-1, is known to be the main substrate for JNK kinase. JNK phosphorylates Ser63 and Ser73 in c-Jun N-

terminal transactivation domain. Numerous reports prove that c-Jun is an essential mediator of cell apoptosis and differentiation (Hall et al., 2000, Prise et al., 1996; Taimor et al., 2001). Other studies indicate the antiapoptotic role of c-Jun (Liebermann et al., 1998; Potapova et al., 2001; Shaulian and Karin, 2001). The induction and activation of c-Jun protein in cells after treatment with chemotherapeutic drugs has been shown in various cell lines (Bironaitė et al., 2003; Shinoda et al., 2005).

The studies of c-Jun protein expression in myogenic cells after their exposure to CisPt, DOX and NOC-18 have revealed a time-dependent increase in c-Jun protein level and in c-Jun phosphorylation (Fig. 12). The phosphorylation of c-Jun was significantly elevated within 8-24 hours after exposure of myogenic cells to CisPt, DOX and NOC-18 and it correlated with the JNK activation after appropriate treatments.

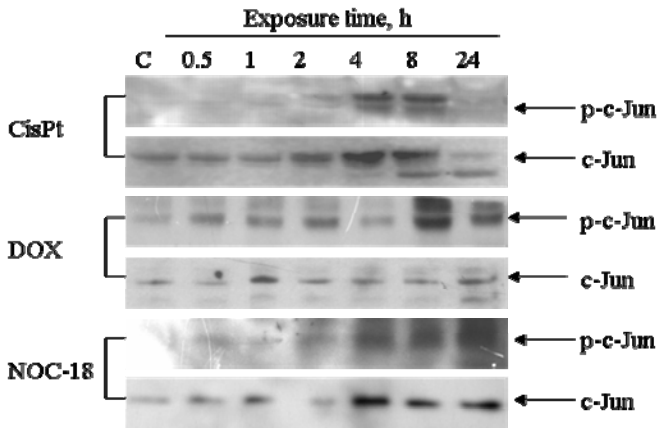


Fig. 12. Time course alteration of c-Jun expression and phosphorylation in myogenic cells. Myogenic cells were exposed to 10 μg/ml CisPt, 2.5 μM DOX; 5 mM NOC-18, and 1 mM H_2O_2 at indicated time points. Results are expressed as the representative images from at least three experiments.

To prove that c-Jun is the target of JNK in myogenic cells we investigated the c-Jun phosphorylation after JNK-specific SP600125 inhibition. We found that JNK inhibition decreases c-Jun phosphorylation and c-Jun expression (Fig. 13). The literature data about the presence of AP-1 binding sites within *c-jun* promoter suggest that transcription of *c-jun* could be subject of a positive autoregulatory loop (Angel and Karin, 1991).

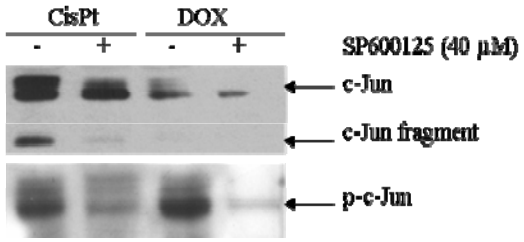
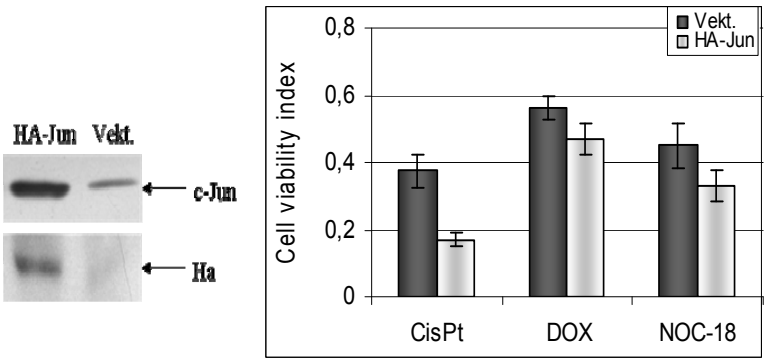


Fig. 13. The effect of JNK inhibitor SP200125 on c-Jun expression and phosphorylation. Myogenic cells were pretreated with SP600125 (40 μM) for 30 min before adding CisPt and DOX. c-Jun expression and phosphorylation were registered by Western blot after 8 h exposure. Results are expressed as the representative images from at least three experiments.

The time-course of the induction of c-Jun phosphorylation strongly suggests its involvement in apoptotic signaling after cardiotoxic treatments. Thus, the proapoptotic functions of JNK after CisPt and DOX treatment are likely to be mediated by c-Jun.

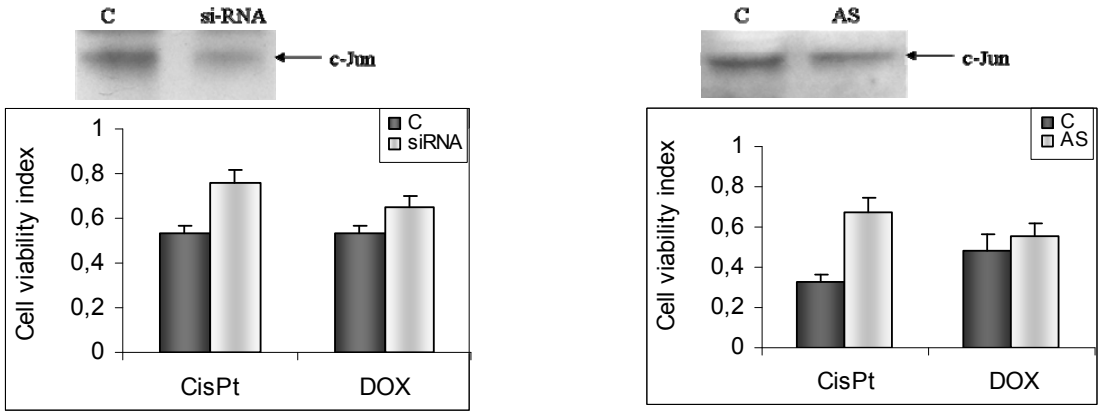
To asses the role of c-Jun, we used genetically modified myogenic cells with increased c-Jun expression, which was achieved by stable transfection of pcDNA-HA-Jun plasmid into myogenic cells. The increased c-Jun expression was confirmed by Western blot analysis (Fig. 14, A). The results obtained revealed that pcDNA-HA-Jun transfected cells (HA-Jun cell line) were more sensitive to treatments with cardiotoxic agents than cells transfected with empty plasmid (Fig. 14, B). These results suggest the involvement of c-Jun transcription factor in the induction of apoptosis in myogenic cells.



A B

Fig. 14. The role of c-Jun in myogenic cell apoptosis induced by cardiotoxic treatments. (A) – Expression of c-Jun and HA in pcDNA-HA-Jun (HA-Jun) and empty plasmid (Vekt.) transfected cells was registered by Western blot. (B) – Ha-Jun and control (Vekt.) cells were treated with CisPt (10 μg/ml), DOX (2,5 μM), and NOC-18 (5 mM) for 24 h. Results are expressed as ratio of viable cells after 24 hours exposure to viable cells before treatment. Data are expressed as mean ± SD from at least three measurements; in all cases $P<0.05$.

A possible c-Jun proapototic role was proved using small interfering RNA (siRNA). The results obtained showed that c-Jun-si-RNA transfected myogenic cells were more resistant to CisPt and DOX than control cells (Fig. 15, A). The decreased c-Jun protein level was confirmed by immunoblotting. Similar results were also obtained on genetically modified myogenic cells transfected with eukaryotic expression vectors carrying *c-jun* in antisense orientation (Fig. 15, B). Thus, the results show the proapoptotic role of c-Jun in myogenic cell apoptosis induced by cardiotoxic treatments.



A B

Fig. 15. The role of c-Jun in myogenic cell apoptosis induced by cardiotoxic treatments. siRNR transfected cells (A) and cells transfected with *c-jun* in antisence orientation (B) as well as control cells were treated with CisPt (10 μg/ml, 24 h) and DOX (2,5 μM, 24 h). Results are expressed as ratio of viable cells after exposure against viable cells before treatment. Data are expressed as mean ± SD from at least three measurements, the significance of results with CisPt and DOX treated myogenic cells was $P<0.05$ and $P<0.02$, respectively. The decreased c-Jun expression was evaluated by Western blot.

In conclusion, we can state that JNK may contribute to apoptotic signaling via c-Jun/AP-1 in CisPt- and DOX-induced apoptosis. One of the targets of c-Jun is known to be Fas ligand (FasL).The transcriptional activation of FasL leading cells to the apoptosis is commonly interrelated with sustained JNK/c-Jun activation (Mansoury et al., 2003). Thus, we investigated the expression of FasL after exposure of cells to CisPt, DOX and NOC-18. The obtained data did not reveal any changes in the FasL protein level (Fig. 16, A). However, use of antibodies simulating the FasL action showed that FasL/Fas

pathway is involved in CisPt-induced myogenic cell apoptosis (Fig. 16, B). As reported in literature, the expression of Fas itself is regulated by JNK-c-Jun pathway.

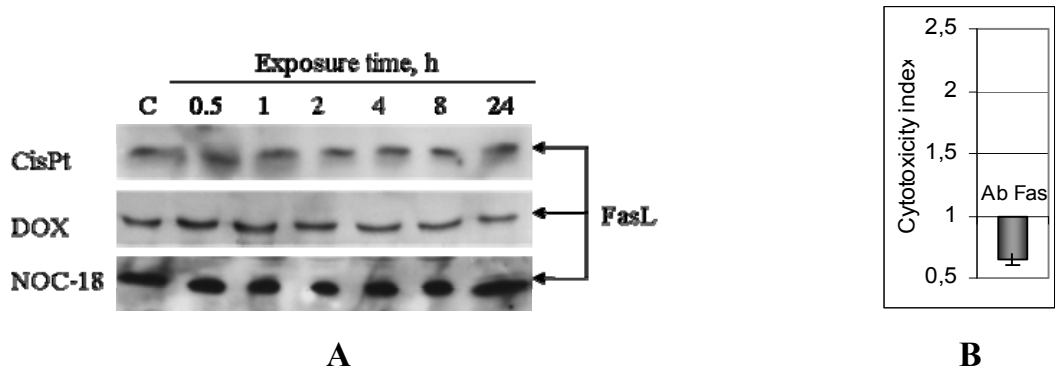


Fig. 16. The role of FasL/Fas pathway in myogenic cell apoptosis. (A) – Expression of FasL after treatment with CisPt (10 μg/ml), DOX (2,5 μM), NOC-18 (5 mM) at indicated time points was registered by Western blotting. (B) – Myogenic cells were preatreated with antibodies against Fas for 30 min before adding CisPt. Cytotoxicity index express the ratio of viable cell treated with anti-Fas antibodies and CisPt simultaneously to viable cells exposed to CisPt. The effect was registered after 24 h exposure. Data are expressed as mean ± SD from at least three measurements, $P<0.03$.

The role of PI3K/Akt signaling pathway in myogenic cell apoptosis. Numerous reports have documented the protective role of PI3K/Akt signaling pathway in various cells. It was shown, that the PI3K, protein kinase C (PKC), and extracellular signal-regulated kinase (ERK) signaling pathways protect the heart against ischemia and reperfusion injury (Cai ir Semenza, 2007). Akt/PKB protein is implicated in prevention of skeletal muscle and cardiac cells apoptosis induced by various stresses (Fujio et al., 1999; Negoro et al., 2001). Akt is known to mediate cell survival by regulating several effectors, including Bad, procaspase-9, GSK3 and transcription factor FOXO (Manning & Cantley, 2007). Another substrate for PI3K/Akt is $P70^{S6K}$, which is known to be a physiological kinase for the ribosomal S6 protein whose phosphorylation increases the rate of initiation and translation of mRNA by ribosomes (Van Empel et al., 2004). It is

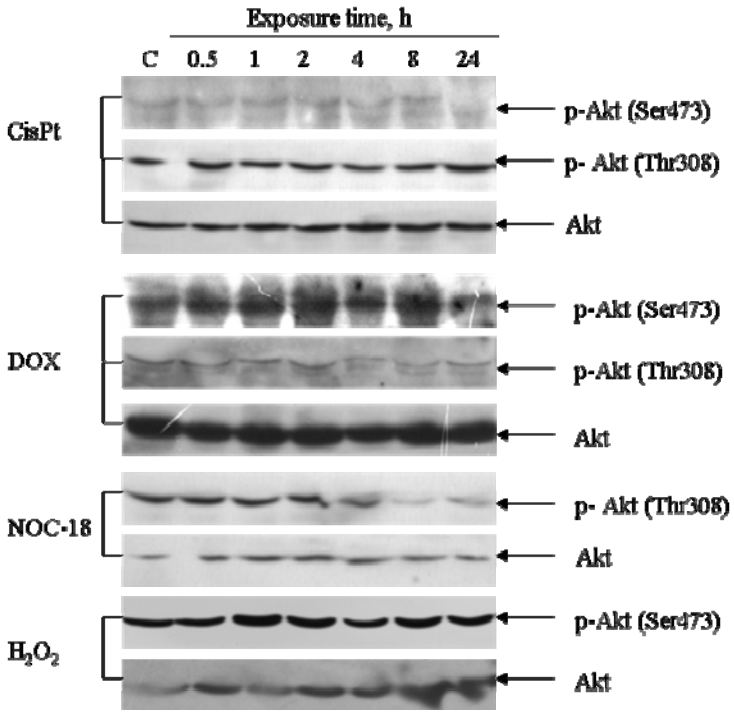


Fig. 17. Time course analysis of Akt expression and phosphorylation in myogenic cells. Tested cells were treated with CisPt (10 μg/ml), DOX (2,5 μM), NOC-18 (5 mM) and H_2O_2 (1 mM) at indicated time points and analyzed by Western blot. The results are representative of at least three experiments. C is untreated control.

possible that Akt exerts its antiapoptotic effects through the crosstalk with other signaling pathways.

The data obtained by us did not reveal any noticeable changes in Akt expression and phosphorylation status after the treatment with cardiotoxic agents (Fig. 17). There was shown constitutive Akt phosphorylation in myogenic cells, which slightly decreased after prolonged exposure to CisPt, DOX and NOC-18. In order to evaluate the degree of Akt phosphorylation in our cells, we compared the expression and phosphorylation of Akt in various myogenic stem cell lines and in the inducible system. It is known that PDGF growth factor induces a marked increase in phospho-Akt protein level in mouse NIH 3T3 cells (Hiromura et al., 2004). The data presented in Fig. 18 show the high basal level of Akt phosphorylation in myogenic cells. Thus, Akt is constitutively activated in muscle derived stem cell lines.

Glycogen synthase kinase-3 (GSK-3) is known to be the downstream Akt kinase target. It is well established that active Akt phosphorylates and deactivates GSK-3. The differential modulation of the Akt/GSK-3 signaling pathway leads to cytoprotective or proapoptotic signaling responses. It was shown that oxidative stress inactivates Akt and activates GSK-3 resulting in the cell death (Nair and Olanow, 2008). The studies of GSK-3 phosphorylation in myogenic cells neither revealed any changes after the treatment with cardiotoxic agents. The data presented in Fig.19 show the constitutive GSK-3 phosphorylation after CisPt, DOX and NOC-18 treatment.

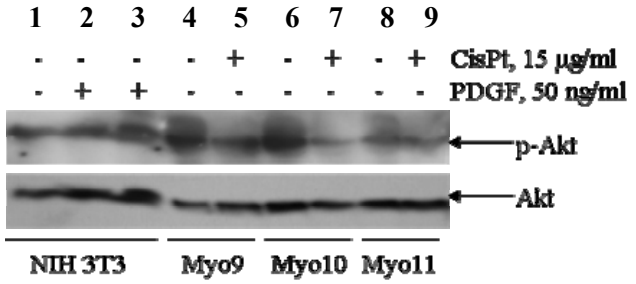


Fig. 18. The level of Akt and phospho-Akt proteins in myogenic cells. The expression and phosphorylation (pThr 308) were analyzed in untreated and CisPt-treated Myo9, 10, and 11 cell lines. Cells were exposed to CisPt for 24 h. NIH 3T3 cells were used as control cells. The expression of Akt in these cells was induced by PDGF. NIH 3T3 were untreated and treated with PDGF for 5 and 15 min. (1, 2 and 3 lanes, respectively).

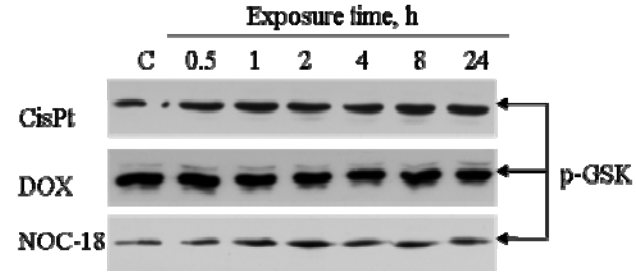
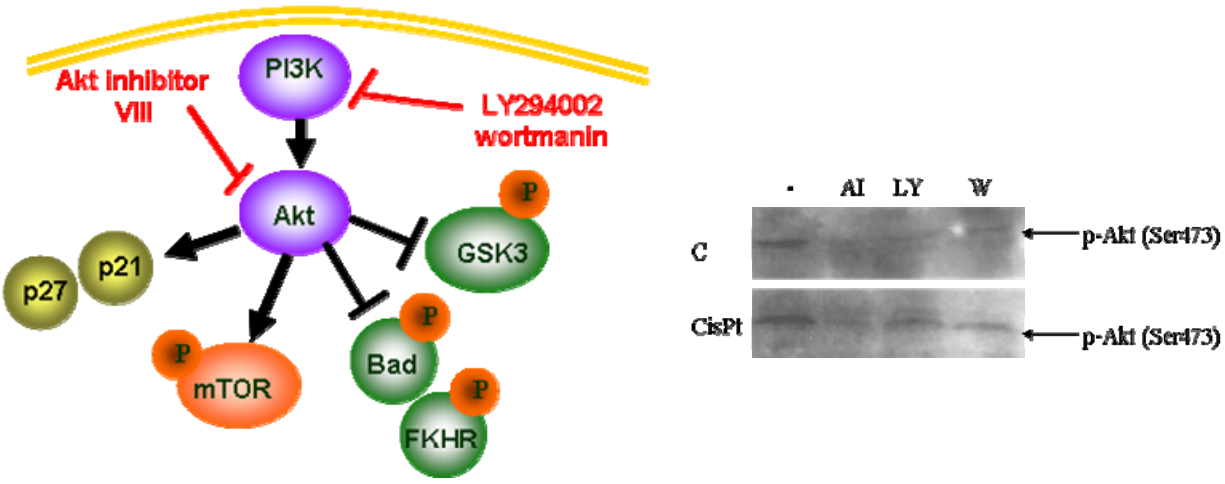


Fig. 19. The activation status of GSK-3 in myogenic cells. Cells were treated with CisPt (10 μg/ml), DOX (2,5 μM) and NOC-18 (5 mM) at indicated time points. GSK-3 phosphorylation was analyzed by Western blot. The results are representative of at least three experiments. C is untreated control.

Moreover, there was also studied the role of PI3K/Akt signaling pathway in myogenic cell apoptosis. For this purpose we used specific inhibitors of PI3K/Akt signaling pathway: PI3 kinase was inhibited by wortmanin and/or LY294002, and Akt kinase, the latter by its inhibitor VIII (AI VIII) (Fig. 20, A). These inhibitors have been reported to increase apoptosis in cancer cells after chemotherapeutic treatments. The effect of inhibitors on Akt phosphorylation in myogenic cells was proved by immunobloting. As shown in Fig 20, B, the inhibitors of PI3 and Akt kinases decreased Akt phosphorylation in control and CisPt treated cells.

Our data indicate that the inhibition of PI3K/Akt signaling pathway by wortmanin, LY294002, and AI VIII sensitized myogenic cells to DOX treatment.

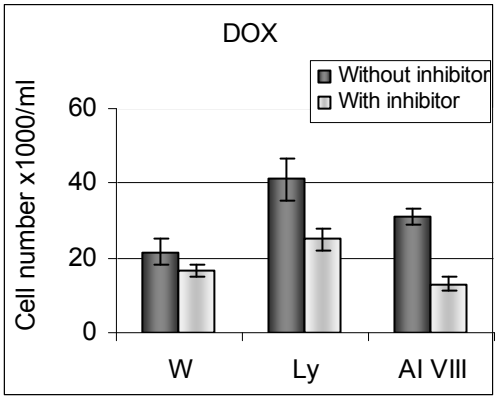


A **B**

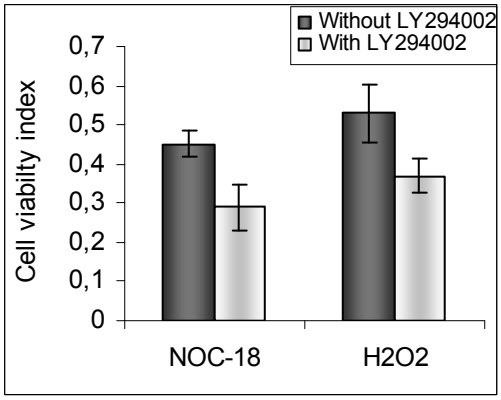
Fig. 20. PI3K/Akt signaling pathway. (A) – PI3K/Akt signaling pathway and its inhibitors. (B) – The action of PI3K and Akt kinases inhibitors wormanin, LY294002 and AI VIII was proved by immunobloting. Cells were pretreated with inhibitors for 30 min before adding 10 μg/mL of CisPt. C – Control, CisPt untreated cells.

Similar results were obtained after exposure myogenic cells to NOC-18, and H_2O_2. PI3 kinase inhibitor Ly294002 enhanced cell death induced by these patophysiogical agents, indicating an antipoptotic role of PI3K/Akt signaling pathway (Fig.21, A and B). However, the inhibition of PI3K/Akt signaling pathway increased myogenic cell survival after their exposure to CisPt (Fig.21, C). It was an unexpected finding suggesting a proapoptotic role of Akt during CisPt-induced cytotoxicity.

A



B



C

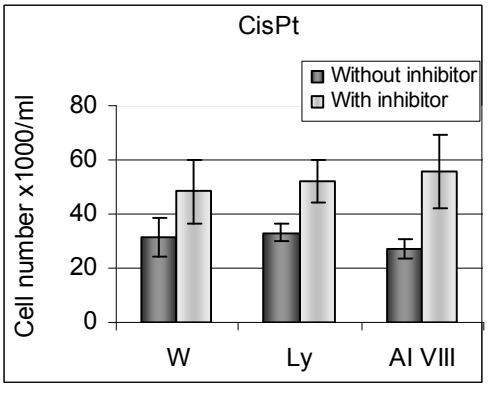


Fig. 21. The role of PI3/Akt signaling pathway in myogenic cell apoptosis induced by cardiotoxic treatments. (A) – The inhibition of PI3 and Akt kinases by its inhibitors increased DOX-induced myogenic cell apoptosis. (B) – Use of PI3 kinase inhibitor LY294002 sensitized myogenic cells to NOC-18 and H_2O_2. (C) – Cells with inhibited PI3K/Akt signaling pathway were more resistant to CisPt. In all cases, cells were pretreated by wortmanin (10 μM), LY294002 (20 μM) and AI VIII (10 μ) for 30 min before adding CisPt (10 μg/mL), DOX (2.5 μM), NOC-18 (5 mM) and H_2O_2 (1 mM). Results are expressed as total cell number ofviable cells (A and C) or cell viability index which was described in previous sections.

Moreover, to confirm a possible proapoptotic Akt action we used genetic methods in order to increase the Akt protein level. Myogenic cells were transfected with puro-Babe-Akt and puro-Babe-vekt plasmids. The established genetically modified myogenic cell lines revealed an increased Akt expression level in comparison to control cells transfected with puro-Babe-vekt plasmid (Fig. 22, A). The genetically modified cells were exposed to cardiotocix agents. The results confirmed previously obtained data. Myogenic cells overexpressing Akt kinase were more susceptible to CisPt than control cells and on the contrary, the resistance of these cells to DOX, NOC-18, and $H_2O_2$was increased (Fig. 22, B).

Thus, the data proved the protective role of PI3K/Akt kinase after genotoxic/cardiotoxic treatment. Furthermore, the obtained results show, that depending on stimulus, PI3K/Akt signaling pathway might promote apoptosis in myogenic stem cells.

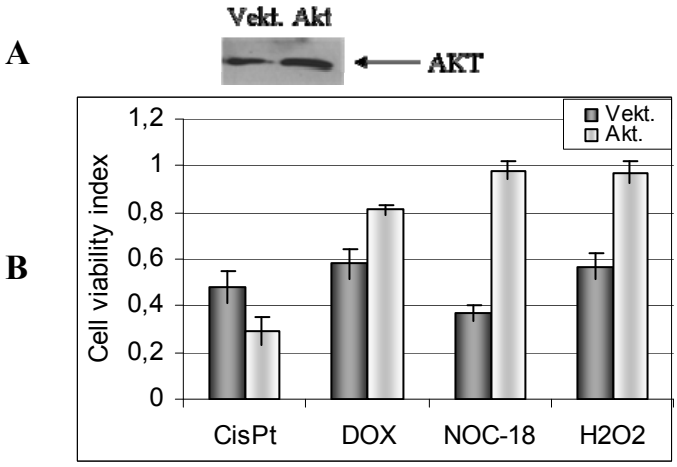


Fig. 22. The role of Akt in myogenic cell apoptosis induced by cardiotoxic agents. (A) – Expression of Akt in myogenic cells transfected with puroBabe-Vekt (Vekt.) and puroBabe-Akt (Akt) plasmids was analyzed and compared by Western blot. (B) – Genetically modified Vekt. and Akt cells were treated with CisPt (10 μg/ml), DOX (2,5 μM), NOC-18 (5 mM) bei H_2O_2 for 24 h. Cell viability index express a ratio of viable cells after 24-hour exposure to viable cells before treatment. Data are expressed as mean ± SD from at least three measurements; in all cases $P<0.05$.

Akt signaling pathway is known to play an important role in the development and progression of cancer and can be also involved in the development of tumor cell resistance to CisPt (Gagnon et al., 2004). Therefore, we compared the role of this signaling pathway in various tumor and myogenic cell lines. The response to CisPt after inhibition of PI3 kinase by LY294002 was analyzed. The obtained data indicated the protective role of PI3K/Akt signaling in tumor cells while in myogenic cells its role appeared to be proapoptotic (Fig.23).

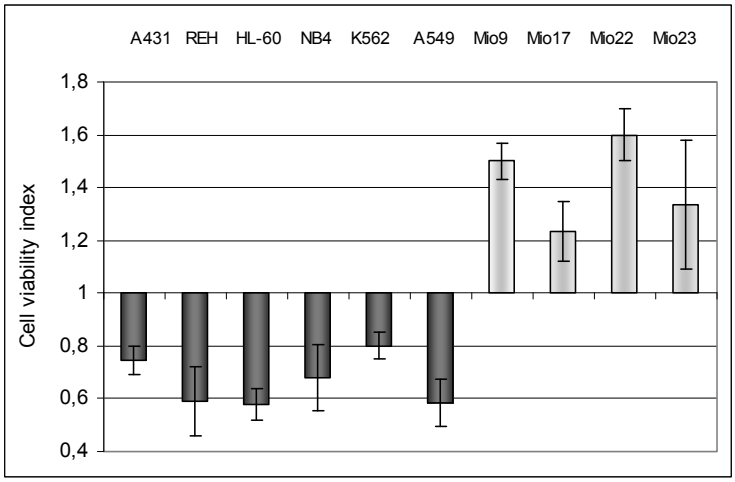


Fig. 23. A comparative study of the role of PI3K/Akt signaling in tumor and myogenic cell apoptosis induced by CisPt. Cells were pretreated with PI3 kinase inhibitor LY294002 for 30 min before adding CisPt. The effect was registered after 24 h exposure. Cell viability index express a ratio of viable cells treated with Ly294002 and CisPt to viable cells exposed to CisPt. Data are expressed as mean ± SD from at least three measurements; $P<0.05$.

In summary, we conclude that ERK, p38, JNK MAPK and Akt kinases may participate in protection of muscle derived stem cells against apoptosis induced by various cardiotoxic agents. The positive modulation (upregulation) of these signaling molecules in transplanted cells might be used for improving the method of cell therapy.

Conclusions

- Adult rabbit skeletal muscle-derived cells demonstrate the properties of stem cells: the possibility of prolonged proliferation *in vitro* and the ability to differentiate along the myogenic lineage.

- Cardiotoxic agents, namely cisplatin, doxorubicin, NO donor NOC-18 and hydrogen peroxide induced myogenic cell apoptosis. In contrast, these cells were resistant to the treatment with inflammatory cytokines TNF-α and IL-6.

- ERK and p38 MAPK signaling pathways are involved in myogenic cell survival after cardiotoxic agent treatments, while the role of JNK in these cells is stimulus-dependent and may be proapoptotic as well as antiapoptotic.

- c-Jun transcription factor, being the main target of JNK, has a proapoptotic role in myogenic cell apoptosis. Thus, JNK contributes to apoptotic signaling via c-Jun/AP-1 in cisplatin- and doxorubicin-induced apoptosis.

- Akt kinase plays a protective role in myogenic cell apoptosis in response to diverse agents, however after exposure to cisplatin PI3K/Akt signaling pathway might promote apoptosis in such stem cells.

- The ERK, JNK, p38 MAPKs and Akt kinases are implicated in the proliferation of myogenic cells. Positive modulation of MAPKs and Akt kinases activity may improve the survival of transplantable cells.

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1. **D. Baltriukiene,** D. Bironaitė, A. Stulpinas, A. Imbrasaitė, V. Bukelskienė, A. Kalvelytė. Mitogenų aktyvinamų kinazių vaidmuo miogeninių ląstelių išgyvenimo/žūties reguliacijoje. Lietuvos Biochemikų Draugijos X suvažiavimas-konferencija. 2008.
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3. Bironaitė D., **D. Baltriukienė**, N. Uralova, V. Bukelskienė, A. Stulpinas, A. Imbrasaitė, A. Kalvelytė. Role of MAP Kinases in Nitric Oxide Induced Muscle-Derived Adult Stem Cell Apoptosis. MOLEDA Summer School "Non-viral gene transfer into muscle and skin"France, 2007
4. **D. Baltriukienė**, V. Bukelskienė, A. Stulpinas, N. Uralova, D. Bironaitė, A. Imbrasaitė, A. Kalvelytė. Role of JNK in muscle-derived stem cell proliferation and apoptosis. International conference "Stem cells and cell therapy", Latvia, 2007.
5. A. Kalvelytė, D. Bironaitė, **D. Baltriukienė**, A. Stulpinas, A. Imbrasaitė, V. Bukelskienė, N. Uralova, J. Šmigelskaitė. Pro-apoptotic Role of Survival PI3K/AKT Signaling Pathway in Muscle-derived Stem Cell Lines. Tarptautinė mokslinė konferencija "Šiuolaikinės Biochemijos iššūkiai ir galimybės", 2007.
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7. Bironaitė, D., Bukelskienė, V., **Baltriukienė, D**., Šmigelskaitė, J., Imbrasaitė, A., Stulpinas, A., Kalvelytė, A. Search of possible of PI3K/Akt pro-apoptotic

8. Bukelskienė, V., **Baltriukienė, D**., Bironaitė, D., Širmenis, R., Balčiūnas, M., Kalvelytė A. (2005) Development of muscle-derived primary cell lines for heart repair. Journal of cardiovascular surgery. Abstracts 46(3) Suppl.1 p.85.

Reziumė

Šio darbo metu gavome pirmąsias suaugusio triušio raumeninės kilmės miogeninių ląstelių linijas, galinčias ilgą laiką augti kultūroje *in vitro* nesikeičiant jų proliferaciniam potencialui. Tai patvirtina šių ląstelių kamieninę prigimtį. Ilgalaikis miogeninių ląstelių auginimas *in vitro* nekeitė ir jų gebėjimo diferencijuotis miogenine linkme. Ilgas auginimas *in vitro* leidžia atlikti genetines šių ląstelių modifikacijas, siekiant padidinti jų atsparumą toksiniams poveikiams, ir tokiu būdu išplečia jų terapinio panaudojimo galimybes. Ikiklinikiniai miokardo regeneracijos kamieninėmis ląstelėmis tyrimai patvirtino šių ląstelių prigijimą išeminiame židinyje. Todėl, mūsų iš suaugusio organizmo raumens gautos ląstelių linijos yra tinkamas ir patogus eksperimentinis modelis ląstelių transplantacijos bei molekulinių kamieninių ląstelių žūties mechanizmų tyrimams.

Pirmą kartą buvo įvertintas miogeninių linijų ląstelių jautrumas galimiems toksiniams poveikiams pažeistoje širdyje bei nustatytas miogeninių ląstelių žūties pobūdis po įvairaus spektro ir įvairių koncentracijų toksinių medžiagų poveikio. Nustatyta, kad ilgalaikis ląstelių auginimas *in vitro* nekeičia jų atsako į šias medžiagas.

Ištyrus miogeninių linijų ląstelių žūties/išgyvenimo mechanizmus nustatytas mitogenų aktyvinamų baltymų kinazių (MAPK) bei Akt kinazės aktyvinimo pobūdis po apoptozės induktorių poveikio bei įvertintas šių signalinių kelių vaidmuo miogeninių ląstelių apoptozės reguliavime. Nustatyta, kad šios signalinės molekulės dalyvauja apsaugant miogenines ląsteles nuo toksinio patofiziologinį kardiotoksiškumą imituojančių medžiagų – NO ir H_2O_2, poveikio. Parodyta, kad streso aktyvinama baltymų kinazė JNK dalyvauja miogeninių ląstelių apoptozės indukcijoje po genotoksinių medžiagų poveikio. Iš kitos pusės, visos šios MAP kinazės yra reikalingos ląstelių proliferacijai. Nustatyta, kad vienas iš pagrindinių JNK taikinių – transkripcijos veiksnys c-Jun, kurį taip pat gali aktyvinti ir kitos MAP kinazės, yra svarbus kardiotoksinių medžiagų sąlygojamai apoptozės indukcijai miogeninėse ląstelėse.

Parodyta, kad PI3K/Akt signalinis kelias didina miogeninių ląstelių atsparumą daugumai kardiotoksinių medžiagų. Tyrimo metu gauti nauji, tirtų kamieninių linijų ląstelėms būdingi, duomenys apie proapoptozinį PI3K/Akt kelio veikimą.

Apibendrinant, mūsų tyrimai parodė, kad MAPK ir Akt signalinio kelio molekulės, dalyvauja kamieninių miogeninių linijų ląstelių žūties/išgyvenimo reguliavime po kardiotoksinių medžiagų poveikio, ir jų aktyvumo moduliavimas ląstelių transplantacijos metu galėtų būti naudojamas tobulinant kardiomioplastikos metodą bei pasirenkant naujas gydymo strategijas.

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Research interests Stem cell, cell signaling, apoptosis

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