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MAPK signalling in skeletal muscle-derived stem cells after daunorubicin treatment

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To shed some light on the cytoxicity of anthracyclines, we developed muscle-derived myogenic stem cell lines (Myo) from different rabbits and examined their susceptibility to various doses of daunorubicin *in vitro*. We have found that myogenic cells after daunorubicin treatment die by apoptosis, a programmed cell death. Stem cells residing in the tissue, after various damages might be protected and even regenerate the damaged organs. With this in mind, we have tested the involvement of MAPKs – ERK, JNK and p38 – in determination of Myo cell fate. We ascertained the gradual and sustained phosphorylation of JNK and p38 by daunorubicin in Myo cells. To evaluate their role in stem cell survival after daunorubicin treatment, we targeted MAP kinases with specific inhibitors. As a result, the JNK inhibitor SP600125 significantly increased cell viability, showing its proapoptotic mode of action. The other MAP kinase, p38, exhibited an antiapoptotic action, while ERK did not show a significant effect on regulating the fate of cells. Therefore, we propose that JNK inhibitors might be used in combination with the chemotherapeutic drug daunorubicin as a complex treatment increasing stem cell survival and preventing the cardiotoxicity of daunorubicin in cancer patients.

Key words: daunorubicin, MAPK, apoptosis, muscle-derived stem cells

INTRODUCTION

Stem cell therapy is a new and promising strategy for the treatment of various disorders. The survival of donor cells in the host environment is an essential part of a successful transplantation therapy. A poor survival of myoblasts after their transplantation into damaged muscle has been described by several researchers' groups [1–3]. Transplanted cells in a damaged tissue experience various stresses and injuries. It is known that inflammation and oxidative stress play a critical role in cardiac injuries such as ischemia / reperfusion and myocardial infarction [4, 5]. Moreover, the transplanted cells in human body must survive for years and could be a target for various kinds of chemotherapeutic drugs, including anticancer compounds.

Activation of apoptotic pathways in normal tissues by chemotherapy is a serious problem. The main concern about the use of anthracyclines, as well as other anticancer drugs, is their cardiotoxicity. Stem cells, as well as cancer cells, in various organs are also subjected to a toxic effect of antineoplastic drugs. It is known that chemotherapy may induce apoptosis in both malignant and normal cells [6, 7]. Therefore, the protection of stem cells from injuries in tissues is a main therapeutical goal. Various approaches, including introduction of *ex-vivo* drug-resistance genes, were developed in order to protect stem cells from the toxic effects of cancer therapies [8].

Whereas cell death / survival pathways are potential targets for improving the efficiency of cell therapy [3], our study has been focused on the understanding of mechanisms determining death / survival processes in adult stem cells. The number of cell lines expressing desmin and able to differentiate into myosin heavy chain-positive muscle cells was established from the rabbit muscle. The stem-cell nature was confirmed by the unlimited proliferative potential of these cells [9].

Daunorubicin, an anthracycline antibiotic and antileukemic drug, has been used in our study as an inducer of apoptotic cell death. It is well known that along with effectiveness in cancer treatments, daunorubicin, as well as other anthracyclines, exert cardiotoxic effects causing serious health problems in cancer patients surviving anthracycline chemotherapy [10–12]. Moreover, changes of cardiac parameters during daunorubicin treatment cause heart muscle degradation and infarction [13]. To reduce the myocardium damage in such patients, the molecular mechanisms of cell death were intensively examined all over the world.

A number of pharmacological drug-action mechanisms have been proposed to explain the alteration of myocardial cell structure and function; much less is known about the regulatory mechanisms leading a cell to death or survival. It is known that apoptosis has been implicated in acute and chronic cardiac diseases [14, 15]. There are reports that anthracyclines, including daunorubicin, cause both apoptotic and necrotic cell death [16–18]. The initiation of programmed cell death by various anticancer drugs activates protein kinase cascades, showing their involvement in apoptosis [19–21]. The investigation of apoptotic or survival signalling pathways has revealed an important role of mitogen-activated protein (MAP) kinases in the fate of cardiac cells [21, 22]. Myocardial MAPKs can be activated by

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ischemia / reperfusion and at the same time might be important in the evolution of ischemic injury [23, 24]. Today, it is known that various heart damages, such as hypertrophy, remodelling of left ventricle, ischemia / reperfussion injury, angiogenesis and atherogenesis also arise from a disbalance of protein kinase signaling pathways analogous to anthracycline treatment [25]. The understanding of intracellular signalling events would enable us to protect cardiomyocytes and stem cells from various types of damages and to prolong their survival in pathological areas. Molecules of MAPK signalling pathways represent possible therapeutic targets in almost all cell types [20, 23, 26]. Genetic and pharmacological manipulations regulating activity of MAPK signalling pathway components would be a way to prevent cell death in pathological circumstances.

Therefore, in this study we have analysed the involvement of members of the mitogen-activated protein kinase family in the daunorubicin-induced adult rabbit muscle-derived stem cell apoptosis. Our data suggest that JNK activation mediates while p38 protects myogenic stem cells from daunorubicin-induced apoptosis.

MATERIALS AND METHODS

Materials. Daunorubicin was purchased from Sigma. The JNK inhibitor SP600125 was purchased from Calbiochem, p38 inhibitor SB203580 from Biosource, ERK signalling pathway inhibitor U0126 from Promega, caspase-3 inhibitor zDEDV-fmk from BD Biosciences. For Western blotting, anti-phospho p38, EKR and JNK antibodies were purchased from Cell Signalling Technology Inc., anti p38, EKR and JNK antibodies from Santa Cruz, secondary HRP conjugated antibodies from Rockland Inc. All inhibitors were dissolved in DMSO (99.8%) from Carl Roth GmbH company. The stock of daunorubicin solution (5 mM) was prepared in water.

Cell culture. Myogenic cell lines, Myo2, Myo5 and Myo9, were derived from adult rabbit leg anterior tibial muscle as described in [9]. Cells were cultured in Iscove's Modified Dulbeco medium (Sigma-Aldrich) with 10% of foetal calf serum (Biochrom AG) and antibiotics: penicillin 100 U/ml and streptomycin 100 μg/ml. Cells were passaged twice a week applying a trypsin and EDTA mixture (0.25% and 1 mM, respectively, in PBS). Cells of passage number 20–40 were used for the experiments. Cell viability was determined by the 0.4% trypan blue exclusion test.

Treatments. For daunorubicin toxicity experiments, cells were transferred into 12 well-plates, approx. 70,000 cells per well. After 24 hours, the old medium and unattached cells were removed and a fresh medium was added. The viable cell number was counted in Neubauer's improved counter before (initial control) and 24 hours after the treatment with increasing concentrations of daunorubicin. Caspase-3 and MAPK inhibitors were added 20 min before daunorubicin. Control cells were treated with appropriate amounts of DMSO.

Apoptosis assay. The apoptotic index was determined according to the Mercille and Massie method as described in [9], using two fluorescent dyes: acridine orange (AO) (Sigma) and ethidium bromide (EB) (Sigma). AO was used to characterize chromatin condensation, and EB was employed to characterize 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 non-fragmented nuclei), NA – nonviable apoptotic with fragmented nuclei (bright orange chromatin, highly condensed or fragmented). Chromatin-free cells totally lost their DNA content and exhibited a weak green-orange staining.

Western blot. Cells were lysed in a lysis buffer (10 mM TrisHCl pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 20 μg/ml Aprotinin, 1 mM PMSF, 2 mM orthovanadate) for 30 min on ice at appropriate time moments: 0.5 h, 1 h, 2 h, 4 h, 8 h and 24 h after daunorubicin addition. Lysates were centrifuged, denatured with SDS sample buffer (0.4 M TrisHCl pH 6.8, 20% glycerol, 4% SDS, 2% 2-mercaptoethanol) and heated for 5 min at 95 °C. Samples were electrophoresed using 10% SDS-PAGE, semi-dry transferred to nitrocellulose membrane and blotted according to antibody data sheet instructions. The ECL (enhanced chemiluminescence) picture was obtained applying Pierce ECL reagents and exposing to X-ray film (AGFA).

RESULTS AND DISCUSSION

In our experimental model, Myo cells were treated with 0.5–10 μM of daunorubicin for 24 h. Cell death was monitored by cell counting, morphological changes and caspase-3 cleavage. Daunorubicin induced a dose-dependent decrease of cell viability as determined after 24 h of treatment (Fig. 1, A). The morphological changes of Myo cells after exposure to daunorubicin revealed an apoptotic mode of cell death (Fig. 1, B). Daunorubicin at high concentrations induced necrotic cell death. The inhibitor of caspase-3, zDEVD-fmk attenuated daunorubicin-induced apoptosis (Fig. 2, A). Myo cells exposed to daunorubicin showed cleavage of caspase-3 (Fig. 2, B). The 17 kD proteolytic fragment of caspase-3 was an indicator of caspase-3 activation and apoptosis induction in Myo cells after daunorubicin treatment (Fig. 2, B). Moreover, the cleavage of caspase-3 was a dose-dependent process (Fig. 2, B).

As MAP kinases have been associated with signalling responsible for apoptosis induction after genotoxic drug treatment and other stresses in various tissues both *in vivo* and *in vitro*, we examined whether daunorubicin activates p38, ERK and JNK MAP kinases in Myo cells. The activation of MAPKs was analysed by Western blotting with anti-phospho-specific antibodies as described in Materials and Methods. Two micromoles of daunorubicin induced 50 percent of cell death after 24 h of treatment (Fig. 1, A) and was chosen for the MAPKs activation experiments. To verify the total level of expression of MAP kinases, Western-blotting membranes were probed with anti-JNK, anti-ERK and anti-p38 antibodies. No variation in the total amount of MAPK was detected (Fig. 3). Western blot analysis demonstrated that daunorubicin induced a strong and prolonged JNK phosphorylation (Fig. 3). A similar manner of JNK phosphorylation was found in several Myo cell lines obtained in our laboratory from different rabbits. A much less activation / phosphorylation of p38 kinase was observed in daunorubicin-treated cells (Fig. 3). Finally, no clear induction of ERK phosphorylation was detected

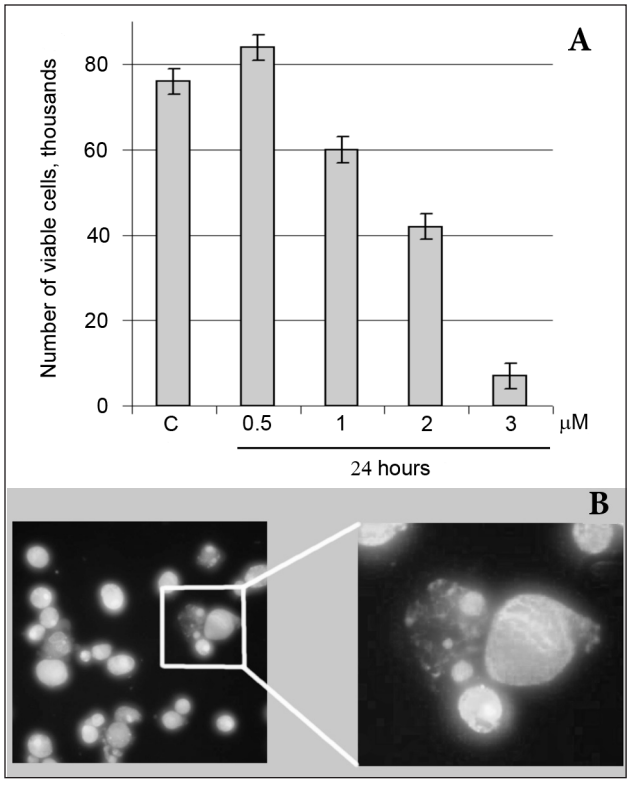

Fig. 1. Daunorubicin induces Myo cell death. **A.** Decreased Myo cell number after daunorubicin treatment. Cells were subjected to various concentrations of daunorubicin, and viable cells were counted 24 hours after treatment. Cell death was determined by trypan blue exclusion test. C – initial control, as described in Matherials and Methods. Data represent means of counted values ± SD from at least four measurements. **B.** Morphological changes characteristic of apoptosis. Myo cells were stained with fluorescent dyes AO / EB as described in Materials and Methods. Figure shows the characteristic morphology of apoptosis (magnified area – viable cell with intact nucleus and apoptotic cell with condensed chromatin and fragmented nucleus). Cells were treated with daunorubicin at a concentration range close to LC_{50}

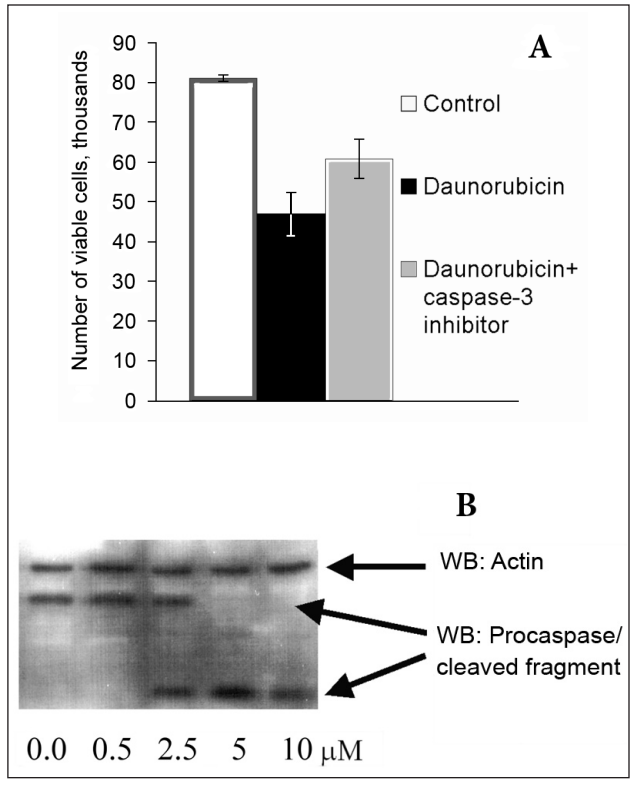

Fig. 2. Participation of caspase-3 in daunorubicin-induced Myo cell apoptosis. **A.** Inhibition of caspase-3 protects Myo cells from daunorubicin-induced cell death. Control cells were treated with 0.2% DMSO only, the second set of cells was treated with 2 μM of daunorubicin together with 0.2% DMSO (Daunorubicin), the third set cells was treated with 20 μM of caspase-3 inhibitor zDEVD-fmk together with daunorubicin (Daunorubicin + caspase-3 inhibitor). Cells were treated for 24 hours, adding DMSO and zDEVD-fmk 20 min prior to daunorubicin. Data represent means of counted values ± SD from at least four measurements. **B.** Western blot (WB) data showing procaspase-3 and its cleaved form after daunorubicin treatment. Beta-actin is a loading control. Caspase-3 activation (specific cleavage) is daunorubicin-dose-dependent

(Fig. 3). Our results indicate that phosphorylation of JNK and p38, but not ERK, was upregulated by daunorubicin (Fig. 3).

To test the biological role of the MAPKs in cell death induced by daunorubicin, we used specific inhibitors of MAP kinases: SB203580 for p38, SP600125 for JNKs and U0126 for the upstream ERK activator MEK1. The cell viability test revealed that JNK participates in the induction of cell death after daunorubicin treatment: the JNK inhibitor, SP600125, effectively attenuated daunorubicin-induced cell death (Fig. 4).

To verify that SP600125 manifests its effect on cell viability by inhibiting JNK activity, we analysed the phosphorylation of the JNK substrate, c-Jun, after treating myogenic cells with daunorubicin. The data showed that c-Jun phosphorylation was decreased by SP600125 pre-treatment (Fig. 5, A). Analogous results were obtained with U0126, an inhibitor of the upstream ERK kinase, MEK1 (Fig. 5, B). ERK inhibition in our system in some cases showed a cell-protective role; however, because of the moderate effect of U0126, further investigations are necessary to clarify the biological role of ERK after daunorubicin treatment. We also analysed the role of p38 MAPK in daunorubicin-induced cell death. In comparison with JNK inhibition results, an opposite effect was found after treating Myo cells with p38 kinase inhibitor:

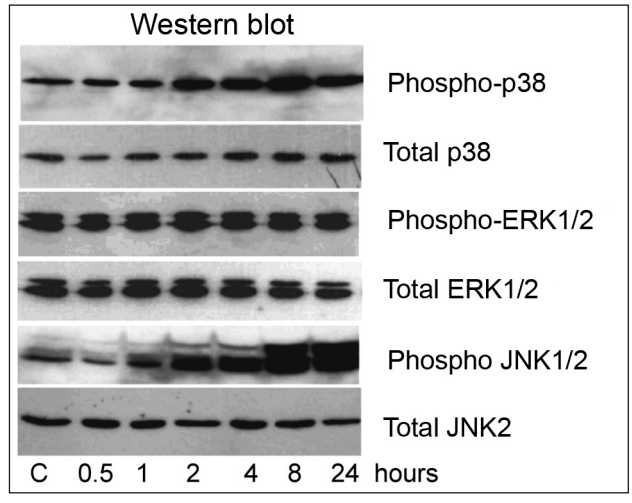

Fig. 3. Phosphorylation of MAP kinases after daunorubicin treatment. p38 and JNK show an activation as determined with anti-phospho-p38 or JNK1/2 antibodies. Immunoblotting with antibodies against total p38, total ERK1/2 and total JNK2 shows no significant changes in protein amounts. In contrast to SAPKs, phospho-ERK is not activated after daunorubicin treatment. Results are representative of three independent experiments

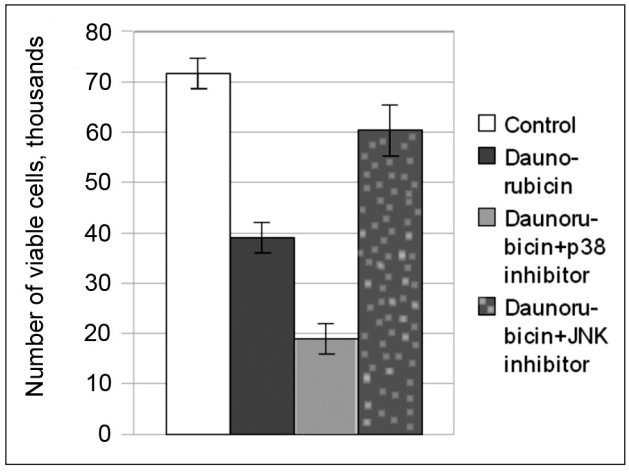

Fig. 4. Biological role of MAPKs in daunorubicin-induced apoptosis. MAPK inhibitors differently influenced cell viability: at a daunorubicin concentration close to LC_{50} (2 μM), the p38 inhibitor SB203580 (20 μM) accelerated cell death, whereas the JNK inhibitor SP600125 (40 μM) protected cells from the toxic effect of daunorubicin 24 h after treatment. Data represent means of counted values ± SD from at least four measurements

SB203580 promoted cell death (Fig. 4). This finding suggests that p38 plays an antiapoptotic role in Myo cells treated with daunorubicin. On the other hand, Engelbrecht and his colleagues have shown the proapoptotic and antiapoptotic role of p38 and JNK, respectively, in neonatal cardiomyocytes [27].

Taken together, we have shown that daunorubicin-induced cell death is mediated via the JNK MAP kinase pathway. The results obtained in this study indicate that long-term activation of JNK is positively related to drug-induced cell death. On the other hand, although the pattern of p38 phosphorylation after daunorubicin treatment was similar to that of JNK, p38 in our cell system plays an antiapoptotic role.

It was suggested that the duration of MAPK activation may be essential in determining their role in cell fate: sustained activation of stress-activated members of MAPKs (SAPKs) is responsible for apoptosis induced by various stimuli, whereas transient activation of SAPKs correlates with cell survival / proliferation. Small changes in the duration of MAPK activation can produce major changes in cell survival [28, 29]. Sustained JNK action was shown to be important as an apoptosis mediator in TNF-alpha-treated cells, and also in cisplatin-induced apoptosis in the ovarian carcinoma cell line [30, 31]. Other researchers' data correlate with our finding that sustained activation of JNK after daunorubicin treatment is proapoptotic. As the early and transient activation of SAPKs usually correlates with cell survival, we analysed p38 phosphorylation shortly after daunorubicin treatment. Our results showed no transient peak of p38 phosphorylation after daunorubicin treatment: we observed a gradual increase of p38 phosphorylation that started early (at 30 min) and reached a maximum 24 hours after daunorubicin treatment (Fig. 6).

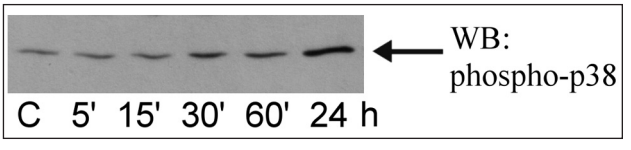

Fig. 6. Pattern of p38 phosphorylation shortly after daunorubicin treatment. Phosphorylation of p38 does not change significantly during the first 1 h of treatment

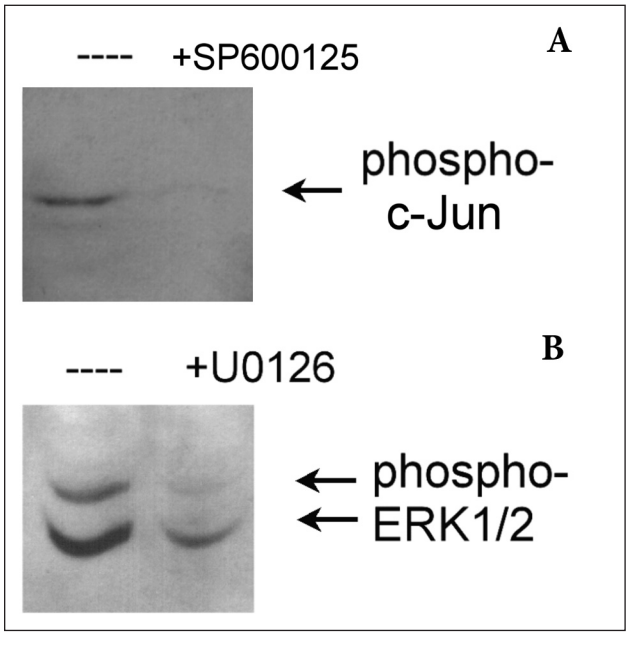

Fig. 5. The blockage of MAPK pathways by specific inhibitors. **A.** Inhibition of c-Jun phosphorylation by the JNK-specific inhibitor SP600125 (40 μM). **B.** Reduction of phospho-ERK1/2 level by the upstream ERK kinase MEK inhibitor U0126 (20 μM)

The involvement of reactive oxygen species was proposed as a mechanism of daunorubicin toxicity [16, 17], although other evidences show that daunorubicin-mediated cardiotoxic effect does not involve free radicals [32]. Free radical cascades induced by daunorubicin and other anthracyclines were proposed as a mechanism for cell death induction. In cardiac cells, anthracyclines were able to trigger a cascade of NO signaling as well [33].

p38 and JNK are members of stress-activated protein kinases (SAPKs). They could be activated in response to oxidative or other stresses. Daunorubicin as well as doxorubicin have been reported to cause activation of SAPKs in cardiac myocytes [16, 18]. One of the ways for ROS-induced JNK activation is the inhibition of their phosphatases [31]. The activation of JNK and p38 MAPK pathways has been suggested to be critical for the induction of apoptosis in most apoptotic systems including heart and muscle cells [19, 21, 34]. The JNK signalling pathway participates in cell death mediated by reactive oxygen and nitrogen species [35]. There are evidences indicating that during oxidative stress-induced apoptosis in adult rat cardiac myocytes, the activation of JNK is crucial for the direct activation of the mitochondrial apoptosis machinery: JNK-induced release of proapoptotic molecules such as cytochrome *c* and AIF from mitochondria by selective permeabilisation of the outer mitochondrial membrane was shown in a cell-free assay [21, 36]. Supposedly, the proapoptotic activity of JNK in mitochondria is executed through the inhibition of antiapoptotic and activation of proapoptotic members of the Bcl-2 family [35].

The other targets of SAPKs in the apoptosis-inducing pathway are the transcription factors p73, c-Jun and others. The JNK and p38 MAP kinase pathways are known as the activity modulators of transcription factors. There are data indicating that the p38 pathway mediates stress-induced transcription of *c-jun*, whereas JNK activates c-Jun protein, a member of the AP-1 (activator protein-1) transcription factors' family [37]. Apoptosis signalling through transcription-dependent mechanisms involves induction of proapoptotic genes [31].

Beside the free radicals, daunorubicin might act by binding directly to DNA as well [38]. Studies indicate DNA damage-triggered activation of MAPKs in DNA damage sensors ATM- and DNA-PK_{cs}-dependent way [39].

Along with the proapoptotic action of SAPKs, there are numerous studies indicating the antiapoptotic role of these kinases. The inhibition of JNK increases apoptosis in the cardiac atrial myocyte cell line exposed to ischemia / reperfusion induced oxidative stress, as well as in C2 skeletal myoblasts treated with TNF-alpha, suggesting the antiapoptotic role of JNK [40, 41]. The antiapoptotic action of SAPKs is exerted via promotion of DNA repair mechanisms or, in cooperation with NF-kB and Akt signalling pathways, by the upregulation of antiapoptotic genes [42, 43].

According to literature data, the role p38 kinase in myogenic cell apoptosis is also controversial – its activation has been implicated in both positive and negative regulation of cell apoptosis. Evidence indicates that p38 is not involved in C2C12 myoblast apoptosis but is required for the apoptosis / anoikis progression of myotubes, induced by $p60^{Fyn}$ inhibition [44].

In summary, daunorubicin activates the phosphorylation of SAP kinases JNK and (to a lesser extent) p38 in myogenic cell lines. According to our results, JNK after daunorubicin treatment is proapoptotic, i. e. is required for apoptosis (prolonged JNK phosphorylation correlates with its proapoptotic action), whereas p38 is an antiapoptotic kinase because its inhibition enhances cell death after daunorubicin treatment.

ERK plays a minor role in daunorubicin-induced myogenic cell apoptosis. Our results indicate that the pattern of MAPK phosphorylation after genotoxic drug treatment not always indicates the cell fate. Anyhow, the manipulation of MAPK pathways for preventing stem cell death is a new approach in cell therapy. Our results suggest that the local application of JNK inhibitors could be advantageous in overcoming the toxic effects of daunorubicin on desirable stem cells during cancer therapy.

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MAPK VAIDMUO APSAUGANT RAUMENINĖS KILMĖS KAMIENINES LĄSTELES NUO DAUNORUBICINO POVEIKIO

Santrauka

Toksiškas daunorubicino poveikis širdies raumens ląstelėms yra pagrindinė priežastis, ribojanti šio efektyvaus priešvėžinio preparato naudojimą pacientams gydyti. Organizmo kamieninės ląstelės taip pat turi būti apsaugotos nuo toksinio chemoterapinių medžiagų poveikio ir išlaikyti regeneracines savybes. Norėdami pagilinti žinias apie daunorubicino toksiškumą ląstelėms, iš triušio raumens išvedėme stabilias kamieninių ląstelių linijas (Myo) ir tyrėme jų atsaką į daunorubiciną. Nustatėme, kad dalis tiriamų ląstelių po daunorubicino žūva dėl apoptozės. Siekdami išsiaiškinti jų žūties mechanizmus, tyrėme trijų MAPK superšeimos kinazių – ERK, JNK ir p38 – vaidmenį. Nustatėme, kad daunorubicinas indukuoja palaipsnį ir ilgalaikį JNK ir p38 fosforilinimo didėjimą Myo ląstelėse. Mūsų modelinėje sistemoje JNK inhibitorius SP600125 efektyviai apsaugo kamienines ląsteles nuo daunorubicino sukeltos apoptozės – taigi JNK dalyvauja Myo ląstelių žūties indukcijoje. Taip pat nustatėme, kad, priešingai JNK, p38 signalinis kelias dalyvauja apsaugant ląsteles nuo daunorubicino indukuotos žūties, o ERK kelias reikšmingo vaidmens neatlieka. Taigi JNK slopinimo, o p38 aktyvinimo strategijos gali būti taikomos siekiant apsaugoti kamienines ląsteles nuo toksinio šio antraciklino poveikio.