Investigation of the mechanism of in vitro stem cell treatment and the role of metabolic memory in oxidative stress induced cellular injury

Ph.D. Thesis

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Introduction

Under physiological conditions, free radical formation and the enzymatic and non-enzymatic antioxidants are in balance, the body is protected from the harmful effects of free radicals. This equilibrium is disturbed under pathological conditions due to the overproduction of free radicals or injury of the antioxidant defense and oxidative/nitrosative stress occurs. Findings from recent years demonstrated that free radicals play an important role in the development of several diseases and their complications such as heart failure, atherosclerosis, ischemic heart disease, hypertension, diabetes, cancer or autoimmune diseases.

The myocardium undergoes several metabolic changes during ischemia/reperfusion, which lead to the destruction of cardiomyocytes and to myocardial infarct. Despite the low oxygen tension during ischemia, moderate reactive oxygen species (ROS) generation is substantiated to occur most probably from mitochondrial source. This is the result of the decrease of ATP level during ischemia when there are no molecular O_2 and nutrients present. Significant ROS production can be observed during reperfusion when reoxygenated blood is flowing through the isolated areas. Reactive oxygen and nitrogen species from the mitochondrial electron transport chain or derived from xanthine-oxidase and NADPH-oxidase play an important role in these processes. Myocardial infarction can be treated using conventional and more recently cell based therapies which were carried out in randomized controlled clinical trials. Cell based therapies combined with the conventional treatment could be a novel therapeutical method in the treatment of heart disease. Recent research intends to identify those cells especially stem cells and factors, which could modulate angiogenesis and formation of cardiomyocytes directly or indirectly at the sites of the injury thus to improve cardiac function. The action of mechanism of transplanted cells is a complex process. Three main mechanisms could be distinguished: transdifferentiation, paracrine factors and cell fusion. More recent studies have reported exchange of cytoplasm and organelles during direct cell-to-cell

interactions. These processes do not appear isolated but rather supporting each other's effects and their distribution is likely to be dependent of the applied cell type and microenvironmental factors of the damaged area.

Elevated blood glucose level in diabetes mellitus is one of the main reasons of cell and tissue damage, which occurs during the early and late complications. Hyperglycemia causes cell and tissue damage through 5 major mechanism: (1) increased flux of glucose and other sugars through the polyol pathway; (2) increased intracellular formation of AGEs (advanced glycation end products); (3) increased expression of the receptors for AGEs and its activating ligands; (4) activation of protein kinase (PK)C isoforms; and (5) overactivity of the hexosamine pathway. The importance of the different metabolic pathways and their role in hyperglycemia induced tissue damage was confirmed by inhibiting certain points of these pathways. For long, it was not clear what is common in these pathways. The unifying process during hyperglycemia is the overproduction of superoxide ($O_2^{\cdot-}$) by the mitochondrial electron transport chain which raises the level of reactive oxygen species. In hyperglycemia, there is more glucose being oxidized in the citric acid cycle, which in effect pushes more electron donors in the electron transport chain, thus the ratio of ATP/ADP increases and the mitochondrial membrane hyperpolarizes. The voltage gradient across the mitochondrial membrane increases until a critical threshold is reached. Electron transfer on complex III is blocked, causing the electrons to back up to coenzyme Q, which transfers electrons to molecular oxygen, thereby producing $O_2^{\cdot-}$. This process is the basis of mitochondrial dysfunction, which is common in the five, above-mentioned pathways and plays an important role in the diabetic metabolic disease. The key glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH) links together the five metabolic pathways. Hyperglycemia induced $O_2^{\cdot-}$ production inhibits GAPDH, levels of all glycolytic intermediates increase and this augments the flux into the five pathways. Modifications of GAPDH by poly(ADP-ribose) polymers (PAR) also inhibit GAPDH activity. PAR polymers and

poly(ADP-ribosylated) proteins are produced by poly(ADP-ribose) polymerase (PARP). Inhibition of PARP enzyme does not affect the activity of GAPDH. When hyperglycemia induced $O_2^{\cdot-}$ production was inhibited with uncoupling protein-1 (UCP-1) or manganese-superoxide dismutase (MnSOD), the activity of GAPDH was not changed and poly(ADP-ribosylation) by PARP was prevented.

The phenomenon of metabolic memory means that early glycemic environment is remembered in the target organs like eye, kidney or heart. What mechanisms could explain the persistence of risk for complications even when hyperglycemia is normalized? It can be assumed that mitochondrial proteins are glycated during hyperglycemia and their function is altered. Levels of methyl-glyoxal (MGO) are increased in diabetes. MGO easily reacts with arginine, lysine, sulfhydril groups of proteins and nucleic acid to form AGE products. MGO has been shown to inhibit mitochondrial respiration and modifies certain mitochondrial proteins. These two processes are in direct contact with each other. Mitochondrial proteins, which are part of the electron transport chain and are glycated, produce $O_2^{\cdot-}$ at normoglycemic conditions.

Aims

1. Our goal was to investigate the mechanisms of cellular damage in ischemia/reperfusion injury in an *in vitro* model. We examined if added, healthy cells can rescue oxidatively injured cells from cell death, which alterations can be detected in the markers of oxidative stress and which mechanisms have a role in the regeneration process. We investigated the effect of paracrine factors, the importance of direct cell-to-cell interactions and the possible role of mitochondria.
2. The effects of hyperglycemia on endothelial cells were examined in an *in vitro* model. We investigated the phenomenon of metabolic memory with the use of fluorescent dyes, which are suitable for detecting oxidative

stress. Alterations in the mitochondrial ROS production and the effect of different inhibitors on these processes were studied.

Materials and methods

***In vitro* ischemia model**

1. H9c2 cardiomyoblast cells were used in these experiments, which were labeled with the fluorescent dye Vybrant DiO (green, 1:200, 30 minutes, 37°C) before simulated ischemia. Ischemia-reperfusion was simulated in vitro by performing oxygen glucose deprivation (OGD) on H9c2 cell cultures for 150 minutes. Vybrant DiD-labeled mesenchymal stem cells (MSCs) (red, 1:200, 30 minutes, 37°C) were added to the damaged H9c2 cells 30 minutes after the end of OGD. Three groups were created: 1) H9c2 cells not receiving MSCs after OGD (control), 2) H9c2 cells cultured directly with MSCs 3) H9c2 cells cultured with MSCs in cell culture inserts (inserts have a membrane pore size of 0.4 μm, physical contact between cells is blocked). Co-cultures were stained with ethidium homodimer 24 hours after ischemia, and then investigated either with confocal microscopy or flow cytometry.

The evaluation of confocal images for live and dead cells selected by morphology and fluorescence was performed with the ImageJ software. In case of co-cultures, MSCs were distinguished from H9c2 cells depending on their Vybrant DiD cell labeling.

Time lapse video microscopy was performed after *in vitro* ischemia to investigate possible interactions (cell fusion and formation of intercellular nanotubes) among the cells over time. In the experiments to observe mitochondria, all cells were stained after OGD with MitoTracker Red in a dilution of 1:2000 for 10 minutes at 37°C.

2. Survival of postischemic H9c2 cardiomyoblasts was investigated after adding healthy H9c2 cardiomyoblasts. The experimental conditions were similar to the experiments with mesenchymal stem cells. Two groups were created: H9c2

cells not receiving H9c2 cells after OGD (control), 2) H9c2 cells after OGD cultured with healthy H9c2 cells (OGD + H9c2).

3. Role of the mitochondria of added cells was investigated using F16, a mitochondrial toxic agent. F16 is a lipophilic cation, which irreversibly inhibits oxidative phosphorylation. Based on preliminary result, added cells were pretreated with 200 μM F16 for 48 hours. These cells were applied as rescue cells beside the groups described in point 2.

4. In case of cellular injury, the integrity of the cell membrane is damaged; enzymes are released from the cytoplasm. Lactate dehydrogenase (LDH) can be found even in case of small membrane damage in the cell culture supernatant. Damage of the plasma membrane i.e. the cell viability can be concluded with the measurement of LDH activity, which was done 24 hours after OGD in the groups described in point 2. LDH activity was measured on 96 well plates, in 100-100 μl supernatants plus the reaction mix. Following 30 min of incubation at 37˚C, activity was measured at 490 nm.

5. Malondialdehyde (MDA) is marker of oxidative stress, the end product of lipid peroxidation. MDA was measured in the groups described in point 2 after adding the rescue cells to the postischemic cardiomyoblasts 5 hours after OGD. Based on the literature, MDA concentration reaches its maximum at this time point. Absorbance was measured at 532 nm on 96 well plate from the calibration solutions and samples.

***In vitro* hyperglycemia model**

Human umbilical vein endothelial (HUVEC) and human microvascular endothelial (HMEC) cells were used in these experiments. Intracellular ROS production was measured with the fluorescent dyes CM-H_2DCFDA and hydroethidine. Three experimental groups were created:

1. HUVECs or HMECs were grown for 3 weeks in low glucose (5 mM, N) containing medium
2. HUVECs or HMECs were grown for 3 weeks in high glucose (30 mM, H) containing medium
3. HUVECs or HMECs were grown for 2 weeks in high glucose, then for 1 week in low glucose containing medium (30→5 mM; H→N)

HUVECs in the 3rd group were treated in the last week with the following inhibitors: 62.5 μM α-lipoic acid (ALA), 10 μM apocynin (APO) or mitochondrial uncoupling protein (UCP2), an antioxidant, inhibitor of mitochondrial ROS production and NADPH-oxidase, respectively. At the end of the experiments, 2 μg/ml CM-H_2DCFDA was added to the cells for 15 minutes at 37°C. Fluorescence intensity was measured with spectrophotometer (excitation/emission: 488/530 nm). The basic phenomenon was investigated on HMECs using confocal microscopy and flow cytometry. Produced ROS was detected with hydroethidine, which was solved in DMSO. Final concentration in the cell culture medium was 1 mg/ml.

Statistical analysis

Results are expressed as mean + SEM ("standard error of the mean"). In case of two groups, Student's t test was used to compare mean values. In case of three or more groups, analysis of variance with Neumann-Keuls post hoc test was applied to compare mean values. Differences were considered significant when $p < 0.05$ (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Results

***In vitro* ischemia model**

1. Confocal microscopy showed that cardiomyoblasts cultured alone displayed the same rounded and blebbed morphology immediately following as

well as 24 hours after OGD. When MSCs were added to postischemic cardiomyoblasts, the morphology of the damaged cells was similar to cells cultured in normal conditions without OGD. Flow cytometry analysis showed that OGD significantly increased the cell death rate in this group as shown by the enhanced ethidium homodimer fluorescence intensity, but added cells decreased the deleterious results of ischemia. To quantify the effect of added MSCs, confocal images were used. This approach revealed that the ratio of dead H9c2 cells to all H9c2 cells in the wells 24 hours after OGD was significantly higher when the cardiomyoblasts were cultured alone compared to when healthy MSCs were added to the cultures 30 minutes after OGD (0.85 ± 0.086 vs. 0.16 ± 0.035, respectively, $p < 0.05$, $n = 5$). MSCs added in cell culture inserts failed to decrease the ratio of dead cells (0.90 ± 0.055, $n = 5$) (Fig 1.).

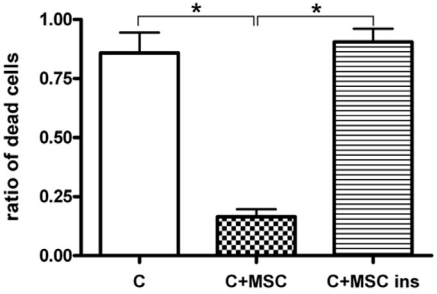

Figure 1. Ratio of dead cardiomyoblasts in the different experimental groups. Data represent mean ± SEM. *p < 0.05 C+MSC vs. C and C+MSC vs. C+MSC ins. (C: H9c2 cells only; C+MSC: H9c2 cells and MSCs; C+MSC ins: H9c2 cells and MSCs in cell culture inserts).

Development of intercellular connections, so-called nanotubes, between cardiomyoblasts and MSCs during the 24 hours period after OGD was frequently observed with video microscopy. These nanotubes were long enough to span distances of several cell diameters, and their diameters were between 200 and 500 nm. MitoTracker Red staining revealed that these nanotubes connecting stem cells to cardiomyoblasts contained functionally active mitochondria. Time lapse video microscopy did not reveal a specific direction for the movement of these mitochondria in the intercellular connections and the typical time frame for the

formation of a nanotube was approximately 2 hours. Addition to the formation of intercellular communications, double labeled and double nuclei cells indicated that cell fusion events were present in the co-cultures. The typical time frame for a cell fusion was approximately 4 hours.

2. Our results show that added, healthy H9c2 also significantly improved the survival of postischemic H9c2 cells after OGD to the same extent as mesenchymal stem cells.

3. H9c2 rescue cardiomyoblasts were pretreated with F16. The number of surviving postischemic H9c2 was as low as in the control group when these pretreated cells were added after OGD, however there were significantly more surviving cells when healthy H9c2 cells were added.

4. Measurement of LDH enzyme activity after OGD showed that added H9c2 cells significantly reduced the number of necrotic H9c2 cells compared to the control group (17.42 ± 2.63 vs. 8.00 ± 2.48, expressed in %), i.e. the added cells reduced the level of cytotoxicity (Fig. 2/A).

5. Levels of MDA concentration in the control group were significantly higher than in the group treated with healthy H9c2 (13.91±0.82 μM vs. 6.53±1.53 μM). We can say that added H9c2 cells reduce the concentration of MDA, which is generated during lipid peroxidation (Fig. 2/B.).

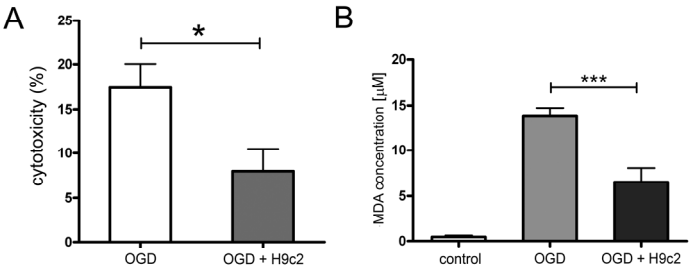

Figure 2. A. Measurement of LDH enzyme activity after ischemia showed that addition of H9c2 cells significantly reduced the level of cytotoxicity compared to control group (17.42 ± 2.63 vs. 8.00 ± 2.48). **B.** Addition of H9c2 cells significantly reduced the amount of MDA, which is generated during lipid peroxidation (13.91±0.82 μM vs. 6.53±1.53 μM). Data represent: mean ± SEM; * $p < 0.05$; *** $p < 0.001$

***In vitro* hyperglycemia model**

Levels of mitochondrial reactive oxygen species and the effect of different inhibitors on these processes were investigated on HUVECs after cells were grown for 2 weeks in high glucose than for 1 week in low glucose containing medium (H→N). We found that the levels of produced ROS remained high in the H→N group compared to the group H. However, addition of the mitochondrial uncoupling protein UCP2, the antioxidant ALA or the NAD(P)H oxidase inhibitor apocynin on the last week of normalized glucose significantly reduced ROS-related CM-H2DCFDA fluorescence but it was unable to return to the level of group N (Fig. 3).

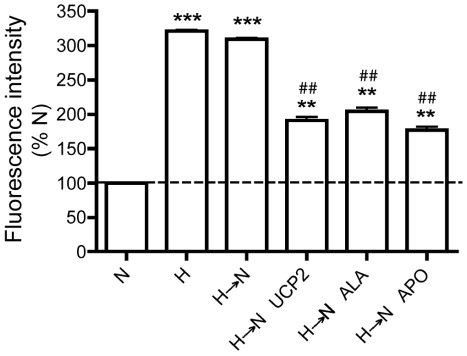

Figure 3. HUVECs were cultured in normal (5 mmol/l; N) or high (30 mmol/l; H) glucose for 3 weeks or for 2 weeks in high (30 mmol/l) glucose followed by normal (5 mmol/l) glucose for 1 week (H→N) with or without 62.5 μmol/l ALA, 25–100 plaqueforming units/cell UCP2 or 10 μmol/l apocynin (APO). Levels of ROS remained significantly high in the group H→N compared to the group H. Addition of the mitochondrial uncoupling protein UCP2, the antioxidant ALA and the NAD(P)H oxidase inhibitor apocynin to the the group H→N significantly reduced ROS-related CM-H2DCFDA fluorescence but it was unable to return to level of group A. Data represent the mean±SEM of the percentage of fluorescence of cells incubated in normal glucose for 3 weeks. **p<0.01; ***p<0.001 vs HUVECs cultured in 5 mmol/l glucose for 3 weeks. ##p<0.01 vs H→N

Measurements with hydroethidine on HMEC cells showed a similar picture as the measurements with CM-H2DCFDA: levels of ROS remained significantly high in the group H→N such as in group H compared to group N.

Discussion

***In vitro* ischemia model**

We investigated the mechanisms of cell damage, which occur during ischemia/reperfusion. H9c2 rat cardiomyoblast cells were used in these *in vitro* experiments. H9c2 cells has been used in previous studies of doxorubicin, glucose-glucose oxidase, hydrogen peroxide or ischemia/reperfusion, therefore it is an accepted *in vitro* model to examine the damage caused by oxidative stress.

Ischemia/reperfusion was simulated by performing oxygen and glucose deprivation on H9c2 cells. Based on preliminary experiments, duration of simulated ischemia was determined at 150 minutes, followed by 30 minutes of reperfusion. In the first part of our experiments, regenerative properties of added, healthy mesenchymal stem cells and the possible mechanism of action were investigated. Postischemic cardiomyoblasts were co-cultured with mesenchymal stem cells for 24 hours. The lowest ratio of dead H9c2 cells compared to the control could be observed when stem cells were added directly to the damaged cardiomyoblasts. Stem cells added in cell culture inserts, which physically separate the two cell population growing in the same medium, failed to decrease the ratio of dead cells. With the use of these inserts, the effects of secreted substances of the mesenchymal stem cells, i.e. the paracrine factors could be examined in the regeneration processes.

We showed that the beneficial effects of co-culture of postischemic cardiomyoblasts and mesenchymal stem cells depend on direct cell-to-cell interactions such as intercellular nanotubes observed with video microscopy. Nanotube formation has already been shown to occur among endothelial progenitor cells, cardiomyocytes, immune cells and other lineages. The characterization of nanotubes revealed that these filaments contain actin and in some cases, microsomes or mitochondria. We found that this phenomenon occurs frequently between cardiomyoblasts and mesenchymal stem cells. MitoTracker Red staining revealed that these nanotubes connecting stem cells to cardiomyoblasts contained

functionally active mitochondria. If rescue cells were pretreated with a mitochondrial toxic agent – called F16 - the rescue effect of the added cells was lost. These results show that mitochondria or parts of mitochondria can get through from one cell to another and could have a role in rescuing postischemic cardiomyoblasts. Direct cell-to-cell interactions are of importance not only after but during ischemia. Video microscopy revealed that cardiomyoblast that were in connection at least with two or three other cells, detached significantly later and died significantly later than cardiomyoblasts without any connection.

One plausible mechanism for the rescuing effect is that transplanted cells improve regeneration through secreting paracrine factors. Conditioned medium of adipose derived stem cells (ASC), which is the collected culture medium of the cells, was shown to have cardioprotective effects. Results from our experiments with the plate insert show that paracrine factors secreted by the cells are probably too low in our system to have any beneficial effect on these severely damaged cells. Co-culturing only for only 24 hours in our system, could be an explanation for the low concentration. Cell fusion is another phenomenon which is frequently observed in co-culture studies and in some cases, in *in vivo* experiments as well. Using videomicroscopy we also found several double labeled, double nuclei cells indicative of cell fusion. However, cell fusion showed high variations among different culture and detection techniques, and therefore extensive cell fusion as an in vitro artefact cannot be ruled out. During our investigations we only observed a few unquestionable cell fusions which cannot account for the rescue of the high number of damaged cardiomyoblasts. It was shown that 20% of the co-cultured, Vybrant labeled cardiomyocytes and adipose derived stem cells fused after 5 days. Other groups have also found that 18% of the co-cultured cells have gone through cell fusion after 4 days. We also found double labeled cells without double nuclei in the co-culture of cardiomyoblasts and stem cells after 24 hours. The double labeling of these cells may be the result of direct cell-to cell connections. During these periods of connection, cells are able to exchange membrane parts and

Vybrant dye molecules can drift from one cell to another. Movement of dye molecules from one cell to another through gap junction connections is precluded because the lipophilic Vybrant dyes are high molecular weight stains and connect to the phospholipid bilayer. Low molecular weight tracers such as calcein-AM get from one cell to another through gap junctions, and high molecular weight tracers by partial cell fusion, thus the conclusion may be drawn that dye transfers after 24 hours in our experiment are most probably the results of direct cell-to-cell connections.

The time frame of the experimental protocol is also important. In our experiments we added the cells at an early time point and terminated the experiment before significant differentiation could occur. It was shown that bone marrow derived stem cells differentiate into cardiomyocyte if the cell types are cultured directly for seven days and the cells can develop direct cell-to-cell interactions. Another group found that mesenchymal stem cells differentiated in co-culture with the growing number of cardiomyocytes in a in a dose-dependent manner. Differentiation was even more pronounced when apoptosis was previously induced in the cardiomyocytes. During a later time point the effect of paracrine factors is probably much more important especially in the differentiation process. These results demonstrate that differentiation does not play a main role in the acute phase of the regeneration. Healthy mesenchymal stem cells are able to improve the survival of postischemic cardiomyoblasts via direct cell-to-cell interaction. This mechanism is not yet implicated in the effects of stem cells after ischemic conditions. The beneficial effect of stem cell grafting may be based not only on improved neovascularisation and replacement of lost cells but on rescuing the damaged cells of the host as well. The most likely explanation of the beneficial effects of MSC co-culture is that these cells improve the chances of the damaged H9c2 cells to restore their function, prevent later cell death and activate the regeneration processes at the site of the injury.

In the following ischemia/reperfusion experiments, H9c2 cells were used as rescue cells instead of mesenchymal stem cells. The added H9c2 cells similar to the mesenchymal stem cells significantly reduced the ratio of dead H9c2 cells compared to the control. In the clinical trials, adipose derived stem cells, cardiac progenitor and stem cells, skeletal myoblasts are used as an alternative cell source beside the bone marrow derived hematopoietic and mesenchymal stem cells because these cells are autologous and can be expanded in large quantities.

We found that addition of rescue H9c2 cells significantly reduced the levels of the oxidative stress markers, LDH and MDA, which are the indicators of membrane integrity and lipid peroxidation, respectively. MDA concentration in the supernatant was three times higher than in the pellet after ischemia, i.e. the produced MDA was released from cytoplasm into the supernatant due to membrane damage during necrosis. This result was also confirmed by measuring LDH enzyme activity. These observations verified the hypothesis that the beneficial effect of the added cells reduces the extent of damage caused by oxidative stress, which may contribute to the regeneration of postischemic cells.

In summary, addition of healthy cells can improve the survival of postischemic cells via direct cell-to-cell interactions and intercellular nanotubes. It is important that healthy rescue cells possess an appropriate energy level.

***In vitro* hyperglycemia model**

We investigated the production of mitochondrial reactive oxygen species in an *in vitro* hyperglycemia model and its role in the development of metabolic memory. The importance of metabolic memory is that following normalizing glucose level after a hyperglycemic period, the production of ROS remains high in the cells, which can lead to the development of diabetes and to its complications. We found that persistence of mitochondrial ROS production occurred in endothelial cells, which were grown for 2 weeks in high glucose, then for 1 week in low glucose, as well as in endothelial cells grown for 3 weeks in high glucose

containing medium. This phenomenon was prevented with various inhibitors, such as α-lipoic acid, UCP2 protein or apocynin, an antioxidant, inhibitor of mitochondrial ROS production and NADPH-oxidase, respectively. The degree of oxidative stress was more pronounced in HUVEC cells that were cultured for 2 weeks in daily changing high and low glucose than for 1 week in low glucose containing medium ("oscillating glucose") than in HUVEC cell grown for 2 weeks in high and for 1 week in low glucose. So the phenomenon of metabolic memory can be observed even after daily changing glucose concentrations. Oscillating blood glucose level causes endothelial dysfunction in patients with type 2 diabetes mellitus.

The phenomenon of metabolic memory was observed in the clinical practice in the 1990s after the evaluation of the results of two randomized controlled clinical trials among type 1 and 2 diabetes patients (DCCT/EDIC and UKPDS) in the 1990s. In both studies, patients received either conventional or intensive treatment. It was shown that the risk of microvascular complication was reduced in patients with intensive glucose control (HbA1c ~7% at the end of the study) compared to the conventional treatment group (HbA1c ~8-9% at the end of the study). However, there was no significant correlation with the decrease of cardiovascular complications. In the 2000s, several clinical studies were initiated to examine the influence of intensive therapy (target HbA1c value <6% at the end of the studies) on the development of cardiovascular complication. The results showed that the mortality was similar or increased in the intensive treatment group compared to the conventionally treated group. Several explanations exist: the number of cases of hypoglycemia has increased in the intensive treated group, weight gain was observed among patients. It is concluded from these results that Hb1Ac target value should be around 7%. This target value appears to be adequate according to the follow up of the clinical trials began in the 1990s. These conflicting results suggest that tight glucose control alone is not enough to fend off the long term evolving complications, but it is also necessary to control non-

glycemic risk factors. It should be noted, however, that beside the metabolic memory, daily blood glucose fluctuations also contribute to the development of complications in the conventionally treated group. Daily blood glucose fluctuations are associated with even more increased oxidative stress than persistent hyperglycemic state in type 2 diabetes patients. This is consistent with the above-mentioned experimental results: increased oxidative stress was observed after glucose oscillation. We can say that besides controlling HbA1c levels with intensive therapy, limiting acute glucose fluctuations may reduce the complications of diabetes. Metabolic memory plays an important role in the development of acute and chronic complications.

Another important issue is to reduce the levels of advanced glycation end (AGE) products and the expression of AGE receptors in addition to restore blood glucose levels because they play a direct role in the development of metabolic memory with the glycation of mitochondrial proteins. Examining patients with type 1 diabetes after conventional or intensive treatment showed that oxidative stress caused by advanced glycation end products was more pronounced in the conventional group compared to the control. This study also suggests that determination of concentration of glycation end products in addition with blood glucose level and HbA1c control can be of diagnostic value.

Conclusion

1. Our results show that added mesenchymal stem cells improve the survival of postischemic cardiac cells. Direct cell-to-cell interactions, such as partial membrane connection or intercellular nanotubes play an important role in these regeneration processes. Mitochondria were observed in the nanotubes between connecting cells. If rescue cells were pretreated with a mitochondrial toxic agent, the survival ratio of postischemic cells was deteriorated. Examining the role of paracrine factors, we can say that the level of secreted substances is probably too low 24 hours after ischemia/reperfusion to have any beneficial effect on these

severely damaged cells. The time frame of the experimental protocol is also important in view the differentiation processes. According to the literature, mesenchymal stem cells differentiate into cardiomyocytes after 5-7 days, so we can exclude the phenomenon of differentiation in our model. We also observed cell fusion between cardiomyoblasts and mesenchymal stem cells. However, according to our data and other data from the literature, the low frequency of cell fusion could not explain all the observed beneficial effect. In our model, the acute regenerative capacity of the added cells can be investigated, while at a later time point the effect of paracrine factors is probably much more important especially in the differentiation process. The beneficial effect of stem cell grafting may be based not only on the replacement of lost cells and improved neovascularisation but on rescuing the damaged cells of the host as well, through the mechanism we described.

2. We showed that levels of LDH and MDA, markers of oxidative stress were elevated after ischemia/reperfusion, which were significantly reduced after addition of healthy cells. In the clinical practice, the transplanted stem cells can also increase the regeneration by eliminating reactive oxygen and nitrogen species, which were generated during ischemia/reperfusion.

3. We demonstrated the phenomenon of metabolic memory on endothelial cells using an *in vitro* hyperglycemia model. ROS production remained high after a hyperglycemic period followed by glucose normalization, which could be reduced with inhibitors but did not return to control level. It is possible that mitochondrial proteins may be glycated, especially proteins of the electron transport chain, which produce more reactive species under normal circumstances and create new AGE products. This is consistent with the results from the clinical trials. In summary, investigation of the metabolic memory and more detailed understanding of the phenomenon are important in the prevention of the early and late complication of diabetes.

Publications

Publications closely related to the thesis

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3. Dr. Kiss Levente, Dongó Eleni, Janicsek Zsófia, Szepes Mónika, Benkő Zsolt, Cselenyák Attila, Dr. Lacza Zsombor: *Őssejtterápia alkalmazásának eredményei perifériás artériás érbetegségben,* Érbetegségek, 2010/03.

4. Book title: “Microscopy: Science, Technology, Applications and Education”, Chapter title: “*Live-Cell Fluorescent Imaging of Membrane or Mitochondrion Transfer between Connected Cells in Culture*, p764-771, Editors: Antonio Méndez-Vilas and Jesús Díaz Álvarez, Volume 1 ISBN (13): 978-84-614-6189-9