The case of normoxia and hypoxia biology essay

Science, Biology

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Abstract

Metformin is anti-diabetic chemical used in the management of type 2 diabetes, but whether its mechanism involves action on the insulin receptor or downstream events is still controversial. In vitro expansion and proliferation of fibroblast stem cells depend on specific environmental conditions, and investigations have demonstrated that one crucial factor is oxygen environment. The major goal of this study was to qualitatively investigate the effect of metformin on the proliferation of skin derived stem cells (fibroblast) in case of normoxia and hypoxia. The effect of metformin on fibroblast proliferation under impact of oxygen tension was studied in vitro. The results suggested that fibroblast proliferation is largely affected by high dose (100 µg/ml) of Metformin, I found that metformin significantly inhibited cell survival by a dose-dependent manner in three doses: Metformin 10 µg/ml, Metformin 50 µg/ml and Metformin 100 µg/ml. Metformin decreased cell survival in dose 100 µg/ml more than other two doses , also the experiment predicts a significant impact of short-term low oxygen 5% oxygen treatment on fibroblast proliferation together with findings that fibroblast exposed to 20% oxygen tension subsequently showed enhanced fibroblast proliferation and viability. By MTT assay results there are no significant changes in the viability of fibroblast derived stem cells during the cell culturing both on the first and the third day after metformin administration. Keyword : Metformin, fibroplast, stem cells, antidiabetic agent

Introduction

Metformin hydrochloride is an oral antihyperglycemic drug in the biguanide class, used in the management of type 2 diabetes (Evans et al., 2005). The primary systemic effect of metformin for the treatment of diabetes mellitus is to increase insulin sensitivity and decrease blood glucose levels through reducing hepatic gluconeogenesis and decreases intestinal absorption of glucose, particularly in peripheral tissues, including skeletal muscles, liver and adipose tissues (Shaw et al., 2005, Bao et al., 2012, Giannarelli et al., 2003). In vitro and in vivo studies have demonstrated the effects of metformin on membrane-related events, including plasma membrane fluidity, plasticity of receptors and transporters (Muller et al, 1997). Evidence is also mounting for its efficacy in gestational diabetes, although safety concerns still preclude its widespread use in this setting (Smoak, 1999). Metformin also used in the treatment of polycystic ovary syndrome, and has been investigated for other diseases where insulin resistance may be an important factor (Holland et al., 2004). Diabetics treated with metformin have reduced cancer risk (Evans et al., 2005), although it is unclear whether metformin affects cancer directly or indirectly by inhibiting the diabetic state (Jiralerspong et al., 2009). Fibroblasts exist in virtually every organ in the human body (Fries et al., 1994). They are defined as adherent cells, which are not endothelium, epithelium or hematopoietic in origin, and have the capacity to synthesize and remodel the extracellular matrix (Parsonage et al., 2005). The main function of fibroblasts is to maintain the structural integrity of connective tissues by continuously secreting precursors of the extracellular matrix (Fries et al., 1994). Fibroblasts secrete the precursors of

all the components of the extracellular matrix, primarily the ground substance and a variety of fibers (Haniffa et al., 2009). The composition of the extracellular matrix determines the physical properties of connective tissues. In addition to their presumed role as scaffolding support, fibroblasts have been directly shown to play roles in regulating self-tolerance, organ development, wound healing, inflammation and fibrosis (Fries et al., 1994, Serhan et al., 2007). Fibroblasts can be isolated using tissue culture adherence from many tissue sites including adipose tissue, placenta, skin, thymus, periosteum, muscle, synovium, synovial fluid, fetal liver and blood, and cord blood (Haniffa et al., 2009. Da Silva Meirelles et al., 2006). Oxygen tension is a crucial parameter for the culture of fibroblast (Carlos et al., 2011). In the human, oxygen concentration varies significantly between the tissues: in the lung parenchyma and in circulation it is comprised between 4% and 14%, the physiological oxygen tension inside the bone marrow is about 5% on average also, there is an oxygen gradient of 1–7% oxygen within the bone marrow depending on the distance to the blood vessels. (Carlos et al., 2011), in other tissues, relatively less irrigated, oxygen concentration is even lower: in the brain, it varies from 0. 5% to 7%, in the eye from 1 to 5% and in the bone marrow from 0% to 4% (Zoran, 2009) . The relationship between proliferation and differentiation rates is a parameter highly influenced by oxygen concentration, which remains to be elucidated. The stem cells are highly sensitive to oxygen, it is an important biochemical signaling molecule for all major aspects of stem cell biology, including self-renewal, proliferation, cell death, differentiation and migration (Grimshaw and Mason, 2000). Oxygen concentrations inside tissues are

considerably lower than atmospheric and oxygen tension commonly used in incubators (Csete, 2005). Moreover, many tissues as cartilage formation during fetal development occurs at low oxygen tension, and chondrocytes in hyaline cartilage are adapted to low oxygen tension (Zscharnack et al., 2008). In articular cartilage, this tension varies between 3 and 10% oxygen at the surface and decreases to less than 1% oxygen in deeper layers (Da Silva Meirelles, et al., 2006). These data suggest that growing stem cells at physiologically relevant low oxygen tension (Fermor et al., 2007), Promote maintenance of embryonic stem cell pluripotent potential and block differentiation. (Li et al., 2009)With the above background, the purposes of this present study were to evaluate the effect of Metformin on proliferation of skin derived stem cells (fibroblast) in the different concentration of oxygen.

Materials and Methods

2. 1 Sample SourcingAfter written consent from the patients, human foreskin samples were obtained by circumcision. The ethics commission of Leipzig University approved the study which was performed in accordance with the Declaration of Helsinki protocols. The study was carried out in the Department of Cell Techniques and Applied Stem Cell Biology, Biotechnological-Biomedical Center, University of Leipzig, Leipzig, Germany.

2. 2 Cell Isolation and CultureFibroblasts were isolated from human foreskin samples. Immediately after circumcision foreskins were placed in sterile phosphate buffered saline (PBS) and stored at 4 °C until use. They were immersed in Betaisodona solution for 20 min and rinsed several times with PBS until they were free of brown staining. After this disinfection the

subcutaneous fatty tissue were removed with scalpel and forceps. Foreskins were finally cut into pieces of about 5 x 5 mm and digested with Dispose II solution (2. 5 mg/ml Dispose II in DMEM) for 3-4 h at 37 °C or alternatively over night at 4 °C. After this enzymatic digestion the skin was washed with PBS and the epidermis was removed from the dermis with forceps. The epidermis was discarded and the dermis was cut in smaller pieces. After that, the dermis was incubated for 2 h with 1 mg/ml collagenase (in PBS with Ca2+ and Mg2+) at 37°C with gently shaking. The pieces of dermis have to be dissolved. The cell suspension was filtered through a 70 µm cell strainer and centrifuged for 5 min at 1800 RPM. The resulting cell pellet was suspended in Dulbecco's Modified Eagle Medium (supplemented with 10 % fetal bovine serum (FCS), 1 % penicillin / streptomycin and Glutamax) and the fibroblasts were cultured in cell culture flasks. Cell numbers were determined by trypan blue exclusion in an improved haemocytometer after each passage. Fibroblasts were analysed for some mesenchymal stem cell markers with flow cytometry analyses. Cell culture experiments were done in 6-well plates (30. 000 cells/well), 12-well plates (20. 000 cells/well) and 24-well plates (10. 000 cells/well). 2. 3 Measurement of cell viabilityThe MTT Cell Proliferation assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT dyes to formazan dyes, giving a purple color. These reductions take place only when reductase enzymes are active, and therefore conversion is often used as a measure of viable cells. A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would

stimulate or inhibit cell viability and growth. All optical density values were measured at 570 nm, which correlates directly with the number of metabolically active cells in the culture. After measurement of absorbance by a Microtiter plate reader (Tecan) with 650- and 570-nm filters, the mean values of approaches (duplicates) were allocated to mean values for the respective control solution (control Metformin– medium without substance). 2. 4 Description of experimentsGrowth in 8-well plates with Metformin in different dilutions, oxygen conditions (normoxia or hypoxia) and time intervals for proliferation analyses (MTT). Dose of Metformin was 100 µg/ml. The Medium was contain DMEM , high Glucose, Natriumpyruvat, Glutamax with 10 % FCS and 1 % penicillin/streptomycin, also 1 ml per well was used. finally, the number of the fibroblast cells was 20000 cells/well. At the first day cell seeding and stimulating with different concentration of Metformin in the next day, then incubated to proceed MTT-test and measure metabolic activity. In our experiment different concentration of oxygen in medium of fibroblast are used. Two plates are used in our experiment, first plate with 20% oxygen, and the second plate with 5% oxygen, MTT-Test is carried at day 1 and 3.

Results

To investigate the effect of metformin on cell proliferation of skin derived stem cells I conducted MTT assay. I found that metformin significantly inhibited cell survival by a dose-dependent manner in three doses: Metformin 10 µg/ml, Metformin 50 µg/ml and Metformin 100 µg/ml. Metformin decreased cell survival in dose 100 µg/ml more than other two doses (Fig. 1) Comparable growth in all wells, differences in proliferation and

percentage of viability (Fig. 2). Figure3. Showed fibroblast stem cells in vitro cultured after isolating with Percoll from skin and morphological changes of fibroblasts by administration of different doses of metformin in day 1 and day 3. At day 3, I can see with increased metformin concentration the number of damaged cells gets also increased, I can see the damaged cells as white clusters Fig 3. E, F. At day 3 the cells are denser than at day 1.

Discussion:

The MTT assay is used to measure the cell proliferation rate and the reduction in cell viability when metabolic events lead to apoptosis or necrosis (Debby et al., 2004). It is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells. The assay is based on the capacity of the cellular mitochondrial dehydrogenase enzyme in living cells to reduce the yellow water soluble substrate dimethylthiazol -diphenyl tetrazolium bromide (MTT) into a dark blue /purple formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cells line (Elisa and Michael, 2011). The results of the MTT assay graphically represented indicate a definite negative influence of Metformin on proliferation and viability of skin derived stem cells during cell culturing both on the first and the third day after administration. With a higher concentration of Metformin 100 µg/ml it led to a correlative decrease in stem cells activity, diagrammed as fig. 1, in the form of absolute measured values OD (optical density) or as fig. 2 in the form of percentage of viability, the activity of fibroblast stem cells increasing by low concentration of Metformin 50 µg/ml, 10 µg/ml respectively. I found only low differences in respective

data between the normoxia and hypoxia conditions, for the last something better for absolute values of day 1, but not for values of day 3, thus show no significant influence of oxidative stress on cell proliferation in this part of experiment. These researchers: Domm et al., 2002; Kurz et al., 2004; Mizuno and Glowacki, 2005. Studying the influence of low oxygen (hypoxia) during the different type cells and fibroblast stem cells proliferation in three dimensional systems. Studies observed beneficial effects of different precursors and articular stem cells, partially controversial results may be caused by the different cell types used and the application of different cultural systems with different oxygen diffusion properties. I focused on the effect of low oxygen on fibroblast proliferation. Fibroblast expanded at physiologically normxia 20% Oxygen was strongly accelerated and enhanced compared to low oxygen tension 5% oxygen. This result was consistently observed by cell viability in MTT test measurements. Comparable results were obtained for cell proliferation in normaxia and hypoxia culture. Oxygen tension has previously been implicated in senescence (Henrotin et al., 2005). On the one hand, high oxygen levels were shown to promote the generation of free-radical-derived reactive oxygen species (Michiels et al., 2002). If reactive oxygen species reach abnormally high concentrations, they can potentially damage DNA, proteins, and lipids (Zscharnack et al., 2008)My findings underline the importance of appropriate fibroblast-culture conditions during proliferation for the application of these cells in regenerative medicine. My results are in agreement with previous data (Sekiya et al., 2002, Li et al., 2009) which demonstrated that the initial monolayer culture conditions for fibroblast proliferation, including seeding densities, media,

culture period and oxygen tension play an important role in determining the fibroblast stem cells proliferation and differentiation. In agreement with works using cells from other species (Grayson et al, 2007: Lennon et al., 2001: Zscharnack et al., 2008), I demonstrated an advantage of 20% oxygen culture for expansion and proliferate stem cells in monolayer culture. Together with findings that fibroblast exposed to 20% oxygen tension subsequently showed enhanced fibroblast differentiation.

Conclusion

The results suggest that stem cells proliferation is largely affected by high dose (100 µg/ml) of Metformin, I found that metformin significantly inhibited cell survival by a dose-dependent manner. Metformin decreased cell survival in dose 100 µg/ml more than other doses , also the experiment predicts a significant impact of short-term low 5% oxygen treatment on fibroblast proliferation together with findings that fibroblast exposed to 20% oxygen tension subsequently showed enhanced fibroblast proliferation and viability. There are no significant changes in the viability of fibroblast on the first and the third day after metformin administration.

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