

Thesis for the Degree of Doctor of Philosophy
(Ph.D.)

Myeloprotective effects of rosiglitazone,
an insulin sensitizer,
on 5-fluorouracil-induced toxicity

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1. INTRODUCTION

Mortality has always been high among patients with malignant diseases, but even today despite all the complex anticancer therapy the mortality rate stands at about 50% within 5 years in cases required systemic treatment. As a result the 2nd leading cause of death following the cardiovascular diseases has become malignancy (WHO report 2003).

Antineoplastic therapy-associated hematopoietic toxicity often results in neutropenia, anemia and thrombocytopenia. Neutropenia specifically has been shown to force dose reductions, cause treatment delays in subsequent chemotherapy cycles, lead to increased opportunistic infections and ultimately reduce survival. The risk of infection and complications is related to both the severity and duration of neutropenia (Kuhn, 2002). In clinical experience, these serious infections will not improve, despite modern antibiotic and antifungal therapy, until the patient's absolute neutrophil count is normalized (Bodey et al., 1994). Chemotherapy-induced hematopoietic toxicity is a multifactorial challenge in the treatment of oncology patients. Hematopoietic growth factors are often used to moderate chemotherapy-induced myelotoxicity.

Insulin and insulin-like growth factors are early-acting growth factors for hematopoietic stem and progenitor cells. They are widely used for hematopoietic cell cultures to support colony formation of hematopoietic progenitor cells alone or together with some other growth factors. In spite of these facts there is no documentation of insulin's effects regarding hematopoiesis *in vivo*. *In vivo* effects of insulin are hindered by effects of insulin like growth factors and insulin's numerous metabolic effects. It appeared that it could enhance the survival of the cells. This is noted through significantly increased number of all progenitors and doubled both the granulocyte-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) recovered from CD34+ early progenitor hematopoietic cells *ex vivo* when compared to their insulin-free counterparts (Ratajczak et al., 1998). In our work we studied effect of insulin on granulopoiesis *in vivo* in mice. However the use of insulin *in vivo* is not considered practical due to insulin's abundant metabolic effects e.g. hypoglycemia, therefore it is much more convenient to use an agent with same properties and more moderate metabolic effects such as insulin sensitizers namely rosiglitazone.

Insulin resistance is the major cause in Non Insulin Dependent Diabetes Mellitus. Thiazolidinediones, used for the treatment of diabetes mellitus type 2, counter insulin resistance by different cellular mechanisms (Bailey, 2005). Thiazolidinediones modulate gene expression by binding to nuclear transcription factor, peroxisome proliferator-activated receptor-gamma (PPAR γ). PPAR γ is expressed in several tissues; therefore, thiazolidinediones have biological effects on multiple organ systems. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptor isoforms with key roles in the regulation of lipid and glucose metabolism. Synthetic ligands for PPAR γ have effects of promoting insulin sensitization in the context of obesity. Although the in vivo mode of action of PPAR- γ activators is not well understood, it is highly expressed in adipocytes and macrophages, suggesting these cells could be important targets. As PPAR γ nuclear receptors frequently form heterodimers with RXR retinoid receptors, it could be suspected that they might play a role in differentiation of these cells. In hematopoiesis, PPAR γ expression is increased during differentiation of monocytes to macrophages and the activation of PPAR γ /RXR-regulated genes induces macrophage differentiation (Nagy et al. 1998). Dysregulation of PPAR γ has been implicated in aberrant differentiation in some leukemia. Ligand-activation of the PPAR γ and RXR α heterodimer in myelomonocytic leukemia cell lines or other myeloid leukemic cells induces changes characteristic of macrophages (Tontonoz et al.1998, Konopleva et al. 2004).

2. AIMS

Our aims were to investigate:

1. In vivo effects of insulin on granulopoiesis in mice with bone marrow damaged by a cytostatic agent, compared to same effects in healthy bone marrow.
2. Whether rosiglitazone has similar effects on normal and damaged bone marrow.
3. If rosiglitazone is able to protect more granulocyte-macrophage progenitor cells during bone marrow damage, and how it can influence the recovery of bone marrow as a function of time.
4. Whether rosiglitazone is able to mitigate the severity of neutropenia during the recovery after cytostatic drug-induced damage.
5. Whether rosiglitazone protects granulocyte-macrophage progenitor cells in bone marrow damaged by repeated doses of 5-FU.
6. Whether rosiglitazone has any effect on plasma insulin and glucose levels.
7. Whether rosiglitazone has any direct effects on progenitor cells of the murine bone marrow *in vitro* and there are any connection with its PPAR γ receptorial effects.
8. Whether rosiglitazone has any direct effects on human stem and progenitor cells originated from peripheral blood of patients after their mobilization from bone marrow before autologous peripheral blood stem cell transplantation.

3. MATERIALS AND METHODS

The present experiments conformed to the European Community guiding principles for the care and use of laboratory animals. The experimental protocol has been approved by the Ethics Committee for Animal Research, University of Debrecen. Management of patients conformed to the Helsinki Declaration. Informed consent and study design were approved by Regional Human Ethics Committee, University of Debrecen.

3.1. Evaluation of bone of bone marrow function

Bone marrow damage was precipitated with a single sublethal dose of 5-fluorouracil (5-FU) intraperitoneally. Mice were pre-treated *in vivo* by the studied agents before the dose of 5-FU. Bone marrow function was evaluated 0, 2, 3, 4, 5, 6, 8, 10 days after the damage in separate experiments in murine groups treated with vehicle or the studied drugs. The decrease in bone marrow cellularity, frequency and content of granulocyte-macrophage progenitors (CFU-GM) determined from bone marrow samples characterized myelotoxicity. Cellularity of femoral bone marrow was calculated from bone marrow cell counts and volumes of the samples. CFU-GM progenitors were grown in special soft-gel methylcellulose cultures. Colonies, defined as groups of at least 50 cells, were counted under a dissecting microscope (SZ6045; Olympus, Hamburg, Germany) at the end of the incubation period. Colonies consisted of granulocytes and/or monocytes, as verified by smears or cytospin preparations.

Frequency of CFU-GM progenitors was established from these methylcellulose cultures. Numbers of colonies grown from 10^5 bone marrow cells showed the intensity of regeneration of CFU-GM pool after the bone marrow damage caused by cytostatic agent. The CFU-GM content of femur (CFU-GM pool) was calculated with the help of the colony numbers and cellularity and was expressed as CFU-GM per femur.

From retroorbital blood samples total white blood cell count (WBC) was counted in haemocytometer, the frequency of neutrophil granulocytes (absolute neutrophil count, ANC) was determined by differential count of 200 cells from blood smears stained with Wright-Giemsa.

3.2. CFU-GM colony assay

The animals were exterminated by cervical dislocation, the femoral bones were aseptically removed. Bone marrow cells were washed out, and single cell suspensions were prepared by suspending them in McCoy's 5A medium (GIBCO Grand Island NY USA) through thin needle syringe. Inocula of 10^5 /ml bone marrow cells were used in petri dishes (Greiner, Nürtingen, Germany) and the murine bone marrow cells were grown in McCoy's 5A modified medium supplemented with amino acids, Na pyruvate, NaHCO_3 , antibiotics (streptomycin, penicillin), 1.2 % methylcellulose and with 25% horse serum. The conditioned medium of WEHI-3B cells containing colony stimulating factors was also added. Cultures were grown in triplicates for 7 days, in a CO_2 incubator (Jouan Co, France) containing humidified atmosphere with 5% CO_2 . Following this the colonies were counted under a dissecting microscope (Olympus, Hamburg, Germany).

3.3. Determining insulin sensitivity

The animals were anesthetized with an initial interaperitoneal dose of 50-mg/kg thiopental-sodium that was repeated as needed. For hyperinsulinemic euglycemic glucose clamping two venous catheters and one arterial cannula were placed in the two external jugular veins, and the right carotid artery respectively. Human regular insulin was infused at a constant rate (20mU/kg, NOVO, Nordisk, Copenhagen) via one of the venous catheters over 120 minutes to yield steady state plasma insulin immunoreactivity of 100 ± 5 $\mu\text{U/ml}$. The glucose infusion rate (mg/kg/min) during the steady state characterized insulin sensitivity (DeFronzo et al., 1979). In the steady state additional blood samples were taken for plasma insulin determination three times at 10 minutes intervals.

3.4. Insulin blood levels

Plasma glucose concentrations were determined in blood samples taken from cardiac puncture of the mice using Accu-Chek (Roche Diagnostics, Mannheim, Germany). Plasma insulin level was measured by means of radioimmunoassay (RIA) using commercially available kits (RK 400 M, Institute of Isotopes, Budapest, Hungary). Both intra- and inter-assay variations were lower than 5%.

3.5. Study design of in vitro experiments

3.5.1. Effects of rosiglitazone on damage of murine CFU-GM progenitors *in vitro*

In vitro effects of rosiglitazone on murine CFU-GM cells were studied in methylcellulose cultures. The femoral bone marrow cell suspension was divided into six portions and cells were grown in the presence of rosiglitazone at a concentration of 1 μ M in the 2nd, 5th and 6th series. On the 5th day of the culturing period 5-fluorouracil was added to the 4th, 5th and 6th series cultures in a 1 mg/l final concentration

3.5.2. Effects of PPAR γ antagonist on rosiglitazone-induced protection *in vitro*

As in previous experiments with except the 3rd and 6th series of cultures in which GW9662, a peroxisome-proliferator-activated receptor-gamma (PPAR γ) antagonist (gift from T. M. Willson, GlaxoSmithKline, Research Triangle Park, NC) was also present from the beginning of the culturing period in a 5 μ M concentration. Cells in the control cultures (1st series) were treated with vehicle in the same manner as the 1st series.

3.5.3. Effects of rosiglitazone on damage of human mobilized peripheral blood stem cells *in vitro*

To detect whether previous beneficial effects of rosiglitazone had a direct or an indirect influence on the hematopoietic cells, in vitro cultures were used. In our experimental system their differentiation into granulocyte-macrophage cells could be studied. Cells were cultured both in presence and absence of rosiglitazone. 5-FU was added to cultures later, on the 5th day.

3.6. Mobilization of bone marrow stem cells and leukapheresis

Bone marrow stem cells were mobilized to the blood with a single 4 g/m^2 dose of Cytosan (Bristol Myers Squibb Co., Princeton, New Jersey USA) and 48 million units granulocyte colony-stimulating factor (G-CSF, Neupogen, Hoffmann-La Roche Ltd., Basel, Switzerland) was administered two times daily from the 3rd through the 10-11th day. Mobilized peripheral blood stem cells were obtained by leukapheresis using a Fresenius Com.Tec system (Fresenius Com.Tec GmbH, Hamburg, Germany). Apheresis was initiated on the 10-11th day in the recovery phase after chemotherapy if the CD34+ cell count was higher than $20/\mu\text{l}$ in the blood. Two- $3 \times 10^8/\text{kg}$ mononuclear cells with $3-4 \times 10^6/\text{kg}$ CD34+ cells were obtained from the patients. Cells were resuspended in 100 ml Iscove's Modified Dulbecco's Medium (IMDM) with 1% human serum albumin and mixed slowly with equal volume of freezing solution containing 5 % dimethyl sulfoxide (DMSO) in final concentration. The samples were then frozen by a computer-controlled cryopreservation system (Cryomed Freezer, Thermo Forma, Marietta, Ohio, USA) at -190°C using liquid nitrogen at the Cell Therapy Laboratory, University of Debrecen. Cells were thawed rapidly in a water bath maintained at 37°C . The total volume of the separated and subsequently thawed cells was used for the autologous transplantation and the rest of the cells were cultured in our experiments.

3.7. Colony formation of human mobilized peripheral blood stem cells

Methylcellulose (Methocel, 3000-5000 centipoise; FLUKA, Buchs, Switzerland) at 1.2% was used as the support matrix for semisolid cultures. McCoy's 5A modified medium was supplemented with amino acids, vitamins, Na-pyruvate, NaHCO_3 , penicillin and streptomycin as well as with $5 \times 10^{-5} \text{ M}$ 2-mercaptoethanol (LOBA, Fischamend, Germany) and 20% fetal bovine serum (Benkó et al., 2000). Mobilized peripheral blood stem cells were separated by Ficoll-Iodamide (Pharmacia, Uppsala, Sweden) gradient centrifugation at $1000g$ for 15 min (specific gravity, 1.077 g/ml). Mononuclear cells from the interphase were washed twice with McCoy's 5A medium containing 5% fetal bovine serum (GIBCO, Grand Island, NY, USA). Using 35mm plastic petri dishes (Greiner, Nürtingen, Germany), 10^5 cells were plated in 1 ml volume of this medium and were incubated for 14 days at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 . Cultures were seeded in triplicates. Cytokines were added to the soft gel cultures just before plating in final concentrations of 300 mg/l for granulocyte colony stimulating factor (G-CSF, Genzyme, Cambridge, England) and of 100 mg/l for granulocyte-macrophage colony stimulating

factor (GM-CSF, Genzyme, Cambridge, England). Rosiglitazone was mixed into the medium and 5-fluorouracil was added to the cultures on the 5th day of the 14-day culturing period.

4. RESULTS

4.1. In vivo effects of insulin pre-treatment on hematopoiesis of healthy and damaged bone marrow

Serious bone marrow damage resulted from application of 5-fluorouracil in 70 and 100 mg/kg doses. The dose-dependent decrease in colony formation of CFU-GM progenitor cells is evident 2 days after 5-fluorouracil administration. When 6 U/kg of long-acting insulin was administered once a day for 5 days before the single 5-fluorouracil dose, the CFU-GM colony numbers grown from 10^5 mononucleated cells were significantly higher than in vehicle-pre-treated groups. Insulin had no effect on healthy CFU-GM progenitors.

4.2. Effect of rosiglitazone on insulin sensitivity in mice

In these experiments we determined the dose range of rosiglitazone, which increased insulin sensitivity in mice. Rosiglitazone applied at oral doses of 6 mg/kg resulted in a significant increase of the insulin sensitivity. The lower doses (1.5 and 3 mg/kg) and the vehicle were without effect.

4.3. Effect of rosiglitazone pre-treatment on 5-fluorouracil-induced myelosuppression

Effect is determined from the comparisons of the 2nd day states of the bone marrow function after the damage caused by 5-FU in the pretreated and non-pretreated groups.

The frequency of CFU-GM progenitors, indicated by the colony numbers grown from 10^5 bone marrow cell inoculates, were higher in samples obtained from mice treated with rosiglitazone and 5-FU than in mice treated with 5-FU alone. This effect of rosiglitazone was dose-dependent. Proliferation of the increased number of progenitors result in an expansion of the CFU-GM pool and 6 mg/kg of rosiglitazone could significantly increase the CFU-GM content after the bone marrow damage ($p < 0.05$).

4.4. Effect of rosiglitazone pre-treatment on vulnerability of CFU-GM progenitors against 5-FU caused toxicity

Effect is determined from the comparisons of the states of the bone marrow function immediately after the damage caused by 5-FU in the pretreated and non-pretreated groups. One hour after injection of a single intraperitoneal dose of 100 mg/kg 5-fluorouracil (5-FU) bone marrow function indicated by the frequency and femoral content of CFU-GM progenitors was less damaged in rosiglitazone pre-treated mice than in mice treated with 5-FU alone ($P < 0.001$).

4.5. Effects of rosiglitazone pre-treatment on recovery of damaged bone marrow after a single dose of 5-FU.

Characterizing bone marrow function by total cellularity, CFU-GM colony formation and CFU-GM content of femoral bone marrow demonstrated that damage to bone marrow function was serious even by the 3rd day following a single dose of 100 mg/kg of 5-fluorouracil. Total cellularity and frequency of CFU-GM cells were 30% and the femoral content of CFU-GM was only 10% of the control value. Pre-treatment with 6 mg/kg rosiglitazone for 5 days resulted in significant improvement of these variables. The total cellularity and the CFU-GM pool increased to 50% of the control values and the frequency of CFU-GM cells was normal even on the 3rd day of regeneration. Recovery of hematopoiesis was very slow after bone marrow damage caused by 5-fluorouracil. By the 6th day cellularity stood at 30% after a nadir of 18% and CFU-GM content came up to only 40 % of the control value despite the elevated intensive proliferation of the CFU-GM cells.

Rosiglitazone had no effect on healthy bone marrow function but recovery of damaged bone marrow was accelerated by rosiglitazone pre-treatment. Higher intensity of proliferation in CFU-GM cells resulted in normal colony numbers as early as day 4 using 3 mg/kg doses and normalization was achieved by day 3 with 6 mg/kg doses. The enhanced proliferation of these groups is highlighted when compared to the group treated by 5-fluorouracil alone where standard colony numbers were reached by day 5. The CFU-GM pool was replenished earlier in rosiglitazone pre-treated groups and was normalized on day 6 using 3 mg/kg doses. In addition, a 2.5-fold higher than normal expansion was observed using 6 mg/kg doses. In the same time frame the group treated with 5-fluorouracil alone reached only 40% of the normal

CFU-GM pool. The nadir of the cellularity was reached one day earlier in the pre-treated groups as opposed to the group treated with 5-fluorouracil alone. These values in pre-treated groups were dose-dependently higher by significant margins.

4.6. Effect of rosiglitazone on recovery of white blood cell counts and absolute neutrophil cell counts after a single dose of 5-FU

The numbers of mature cells originating from hematopoiesis decreased after a latent period in peripheral blood, as the previously formed cells were present. White blood cell counts and absolute neutrophil counts decreased from the 4th day onward. An especially marked slope in levels of absolute neutrophil counts was seen along with a slow recovery phase in the remaining days of the observed period. Mice pre-treated with rosiglitazone had a milder decrease in absolute neutrophil counts, which was also less extensive ($P<0.05-0.001$). Up through the 10th day following 5-fluorouracil administration, the absolute neutrophil counts remained significantly higher than those of the non-pre-treated mice ($P<0.001$).

4.7. Effect of rosiglitazone on granulocyte-macrophage progenitor cells damaged by repeated doses of 5-fluorouracil

To see whether rosiglitazone has myeloprotective effect in bone marrow damage caused by repeated doses of 5-FU mice were randomly assigned into 6 groups. Mice were treated daily for 7 days. Rosiglitazone in 6 mg/kg doses and 5-FU were simultaneously administered to the mice in the combination treatment groups with one-hour intervals between the doses. Following a 7-day-long daily treatment with a combination of intraperitoneal 5-FU and oral rosiglitazone, bone marrow samples were examined for functional evaluation, having allowed only one hour for regeneration after the last 5-FU injection. Cellularity as well as content of the bone marrow in pretreated mice stood significantly higher than the ones only exposed to 5-FU.

4.8. Effect of rosiglitazone on plasma insulin and glucose levels

Insulin levels tend to increase after a 5-day rosiglitazone treatment. In the group pre-treated with 6 mg/kg of rosiglitazone before the 5-fluorouracil dose, a slightly increased plasma insulin was observed together with a significant decrease in plasma glucose compared with the mice treated with 5-fluorouracil alone.

4.9. Effect of rosiglitazone on damage of murine CFU-GM progenitors *in vitro*

Rosiglitazone is a partial agonist on PPAR γ receptors. To detect whether previous beneficial effects of rosiglitazone had a direct or an indirect influence on the hematopoietic cells, *in vitro* cultures were used. The murine femoral bone marrow cell suspension was divided into six portions and cells were grown in the presence of rosiglitazone at a concentration of 1 μ M. On the 5th day of the culturing period 5-fluorouracil was added to the cultures in a 1 mg/l final concentration. As demonstrated pre-treated cultures show a lesser degree of damage and higher number of CFU-GM progenitors preserved.

4.10. Effects of a PPAR γ antagonist on rosiglitazone-induced protection *in vitro*

We investigated whether PPAR γ receptor effects are involved in the rosiglitazone's observed direct protection on CFU-GM progenitors. Neither rosiglitazone nor a PPAR γ receptor antagonist drug (GW9662) affected colony formation of CFU-GM progenitor cells but they influenced their sensitivity to 5-fluorouracil. Colony formation of CFU-GM progenitors after 5-fluorouracil application was increased if they were grown in the presence of rosiglitazone compared with cells cultured without rosiglitazone. This beneficial effect was neutralized by the presence of the PPAR γ antagonist.

4.11. Effects of rosiglitazone on damage of human mobilized peripheral blood stem cells *in vitro*

To detect whether previous beneficial effects of rosiglitazone had a significance in humans *in vitro* cultures of human cells were used. The chosen human mobilized peripheral blood stem cell suspension contained many types of hematopoietic progenitor and stem cells. In our experimental system their differentiation into granulocyte-macrophage cells could be studied, as the grown colonies were especially matured granulocytes and monocytes. Cells were cultured both in presence and absence of rosiglitazone. 5-FU was added to cultures later, on the 5th day. In 1 mg/l concentration 5-FU suppressed colony formation in 87 % of the samples. Rosiglitazone in similar doses than in the case of murine cells protected progenitor and stem cells dose-dependently against 5-FU damage. Colony formation was inhibited only by 42 % in the presence of 0.5 mM of rosiglitazone. One mM of rosiglitazone enhanced differentiation of stem and progenitor cells

5. DISCUSSION

Technical developments and new drugs against malignant cells, as well as the pharmacological modulation of bone marrow toxicity is expected to influence modern chemotherapy for cancer patients. This will hopefully lead to an increase in cure rates with better quality of life.

5.1. In vivo effects of insulin on granulopoiesis in mice with bone marrow damaged by a cytostatic agent, compared to same effects in healthy bone marrow

Our hypothesis was to utilize an early-growth factor like effect of insulin. Although insulin is widely used to support colony formation in cultures of hematopoietic progenitor cells, its effects regarding hematopoiesis *in vivo* have not been documented. Our aim was to study whether insulin itself could influence myelopoiesis *in vivo* in mice.

Insulin had no effects on colony formation of CFU-GM in healthy bone marrow. This may be one of the reason that *in vivo* experiments have been neglected. But it could preserve more progenitor cells in damaged marrow. Hematopoiesis is controlled by a network of cytokine interactions. Many parallel and combined effects converge in hematopoiesis. It is not surprising that neither insulin nor rosiglitazone were able to enhance the optimal colony numbers in balanced normal hematopoiesis in our experiments. But in damaged hematopoiesis they were able to help restore the normal size and ratio of the cell compartments in bone marrow more quickly. Insulin itself has no stimulative effect on progenitor cells *in vitro*, but it can increase their survival (Ratajczak et al., 1998). Equilibrium between survival and apoptosis can be shifted towards survival by insulin (Iida et al., 2002). In the very flexible hematopoietic system some growth factors are able to substitute for each other and many of them can potentiate each other's effects by increasing sensitivity to the subsequent factor during differentiation. By increasing survival and the amplifying the effects of the other colony stimulating factors insulin or rosiglitazone can enlarge the damaged CFU-GM pool and accelerate bone marrow regeneration.

5.2. An insulin sensitizer drug, namely rosiglitazone has similar effects on normal and damaged bone marrow than insulin

After determining insulin sensitizing dose range, similar doses were used in experiments to evaluate whether rosiglitazone has myeloprotective effects. Rosiglitazone similar to insulin has also no effect on colony formation of CFU-GM progenitors in healthy bone marrow, but administered in similar pattern before 5-FU-caused damage it is able to confer protection on bone marrow cells against 5-FU-induced toxicity. The expansion of the CFU-GM pool is required to compensate for the 5-FU-induced loss and allows faster recovery. Granulocyte colony-stimulating factor, which is recently used for facilitating recovery from neutropenia, increases CFU-GM content to twofold in the 5-FU-damaged bone marrow in mice (Gilmore et al., 1995). Rosiglitazone pre-treatment could enhance the CFU-GM content to 3.7fold compared to that in mice treated by 5-FU alone.

We could also evaluate that even immediately after the 5-FU-caused damage were significantly more progenitor cell survived in the pre-treated mice than their 5-FU-treated counterparts. It means that the expansion of CFU-GM compartment based upon at least in part a decreased susceptibility of granulocyte-macrophage progenitors to 5-FU-toxicity *in vivo* by the end of the 5-day rosiglitazone pre-treatment. Rosiglitazone could preserve more progenitor cells for recovery.

5.3. How can rosiglitazone influence the recovery of bone marrow as a function of time?

Such protective effects offer significant clinical application only if a considerable enhancement in restoration of stem cells and facilitation of the recovery phase after injury can be demonstrated. We could state that the non-pre-treated groups reached their intensified regeneration phase following bone marrow damage induced by a cytostatic agent at a later time than groups pre-treated with rosiglitazone. Significant dose-dependent differences were demonstrated by comparing the degree of bone marrow regeneration following 5-fluorouracil injections, as indicated by CFU-GM content and frequency. The high intensity of proliferation in turn resulted in earlier recovery of the CFU-GM pool. Therefore it was logical to conclude that healing started earlier in groups receiving rosiglitazone. Rosiglitazone is not unique in affecting colony formation of some progenitor cells, i.e. granulocyte-macrophage progenitors. It was shown that rosiglitazone could increase colony formation of bone marrow-derived primitive progenitor cells and promote their differentiation towards the endothelial lineage in

mice (Wang et al., 2004). The effective dose range was similar to that which we used in our experiments.

5.4. Whether is rosiglitazone able to mitigate the severity of neutropenia during the recovery of bone marrow after cytostatic drug-induced damage?

The same protective effect was reflected by the absolute neutrophil counts in peripheral circulation. The nadir of absolute neutrophil count was less deep in pre-treated mice and remained significantly higher during the observed period.

The myeloprotective property of rosiglitazone may be due to a range of direct and indirect effects. Some insulin-like effects could be participating in the observed myeloprotection, since the dose range, which increased insulin sensitivity, was similar to that resulting in increased CFU-GM colony numbers. Additionally we have found the expansion of CFU-GM pool a characteristic effect of insulin, in rosiglitazone pre-treated mice. The subcutaneous insulin used in similar treatment protocols also increased the frequency and femoral content of CFU-GM in 5-fluorouracil-damaged bone marrow in mice. *Ex vivo* insulin is added to the culture media when growing and expanding human CD34+ cells (Mobest et al., 1998). Insulin, if added alone to human CD34+ cells cultured under serum free conditions, does not stimulate their proliferation, rather it augments the viability of haematopoietic progenitors by preventing them from undergoing apoptosis (Ratajczak et al., 1998). Rosiglitazone's effects are similar to those of insulin on haematopoietic progenitors. According to Ratajczak et al. insulin rather potentiates stimulatory effects of other growth factors on proliferation of bone marrow progenitors, and it augments their viability in serum-free *in vitro* cultures. Rosiglitazone, similarly to insulin, had no effect on CFU-GM progenitor cell compartment in mice *in vivo*, but could protect it against 5-FU toxicity. Based on these results we feel inclined to believe that rosiglitazone at least in part acts to amplify the effects of insulin on the progenitor cells. Insulin-receptor substrate protein IRS-1 mediates stimulatory effects of insulin in haematopoietic cells shared among some other growth factors e.g. insulin-like growth factor-I (IGF-I) in regulating cell growth and metabolism (Wang et al., 1993, Uddin et al., 1997). Rosiglitazone decreases inhibitory serine phosphorylation of IRS1 *in vitro* and *in vivo* (Jiang et al., 2004).

5.5. Whether is rosiglitazone able to protect granulocyte-macrophage progenitor cells in bone marrow damaged by repeated doses of 5-fluorouracil?

In clinical practice cytostatic drugs are administered over short intensive periods, simultaneous administration of myeloprotective agents would be desirable. To fit protective agents to the treatment schedules of malignant diseases is not easy. Haematopoietic colony-stimulating factors are used frequently to moderate myelotoxicity, but administration of granulocyte colony-stimulating factor G-CSF, the recently used agent supporting bone marrow regeneration, prior to chemotherapy actually may worsen the toxic effects on bone marrow (De Wit et al., 1996). G-CSF has significant beneficial effects only if it is administered after chemotherapy (Timmer-Bonte et al., 2005). This is important in the design of clinical cancer treatment protocols. We expanded our studies to determine whether concurrent use of 5-FU in multiple doses with rosiglitazone influences the observed myeloprotection. Misaki et al. (1998) found that 2-day G-CSF free interval before the single cytostatic dose decreased myelotoxicity while additional pre- and post-treatment with G-CSF even aggravated it.

In contrast to these observations concurrent use of rosiglitazone with 5-FU repeatedly during 7 consecutive days did not unfavourably influence the population of the CFU-GM progenitor cells. One possible explanation is that rosiglitazone itself had no stimulatory effect on progenitor cells even in repeated doses compared with the effects of G-CSF. G-CSF markedly increases both numbers of CFU-GM progenitors and absolute neutrophil counts in healthy volunteers and animals (Goebel et al., 2004, Hernandez-Bernal et al., 2005). Rosiglitazone's effects are similar to those of insulin on hematopoietic progenitors and at least in part it acts to amplify the effects of insulin on the progenitor cells with synergizing stimulatory effects of other growth factors on proliferation of bone marrow progenitors, and increasing their viability.

Rosiglitazone pre-treatment could preserve 15 times more CFU-GM progenitor cells compared to that in mice treated by 5-FU alone and even in the case of simultaneous administration with repeated doses of 5-FU, the expansion of the CFU-GM compartment was 3 times and 50 times greater in the combined-treated mice than in mice treated with 25 and 50 mg/kg repeated doses of 5-FU, respectively.

Although CFU-GM compartment is expanded significantly in combined treated groups when compared with their counterparts treated by repeated 5-FU doses alone, differences in absolute neutrophil counts were not significant. The neutrophil granulocytes disappeared from the blood in majority of cases and despite expansion of CFU-GM progenitor compartment due to rosiglitazone treatment, more time is required for their maturation to granulocytes before the next 5-FU dose. Rosiglitazone with these daily doses might improve neutropenia if longer intervals or shorter cycles are used in administration schedule of 5-FU.

5.6. Whether has rosiglitazone any effect on plasma insulin and glucose levels?

Although hyperinsulinemia is not expected with rosiglitazone treatment (Zawalich et al., 2003, Seda et al., 2002), a tendency toward increased plasma insulin levels was observed in groups treated with 6 mg/kg of rosiglitazone. Some authors published that rosiglitazone can improve insulin secretory responses of pancreatic beta cells to oscillations in plasma glucose levels (Walter and Lubben, 2005). The effect of this small-scale release of insulin on hematopoietic cells cannot be excluded but the more significant myeloprotection suggests the possibility of other influences. A number of other indirect and direct effects could also alter hematopoiesis. Rosiglitazone influences the production of adipocyte-derived factors. These changes may also increase the insulin sensitivity of cells, but some adipokines may also directly regulate myelopoiesis (Bennett et al., 1996).

5.7. Whether has rosiglitazone any direct effects on progenitor cells of the bone marrow *in vitro* and are there any connection with its PPAR γ receptorial effects?

To answer the question whether direct effects on progenitor cells are involved, we studied *in vitro* cultures of these cells. Similar effects were observed in the *in vitro* bone marrow cell cultures than *in vivo*. Murine CFU-GM progenitor cells growing in the presence of rosiglitazone were less damaged by 5-FU. Therefore we concluded that the effects of rosiglitazone on bone marrow progenitor cells were direct. PPAR γ receptors are found in many haematopoietic cell lines (Greene et al., 2000) and its role has been investigated. We found that a PPAR γ antagonist (GW9662) could shield the protective effect of rosiglitazone against 5-FU toxicity to CFU-GM colony formation *in vitro*.

- 5.8. Whether has rosiglitazone any direct effects on human stem and progenitor cells originated from peripheral blood of patients after their mobilization from bone marrow before autologous peripheral blood stem cell transplantation?

Autologous peripheral blood stem cell transplantation is now routinely used for patients with certain hematologic malignancies. After mobilization, blood is enriched with a variety of hematopoietic stem and progenitor cells, which can be obtained by leukapheresis. The damage caused by 5-fluorouracil decreased in a dose-dependent fashion in cultures containing rosiglitazone. Rosiglitazone's beneficial modifying effect was observed in the similar dose range on the human progenitor cells, than on the murine CFU-GM, which raises hope for the future use in practice.

Summarizing our new results:

1. Although insulin is widely used to support colony formation in cultures of hematopoietic progenitor cells, its effects regarding hematopoiesis *in vivo* have not been documented. Insulin had no effects on colony formation of CFU-GM in healthy bone marrow, but it can preserve more progenitor cells in damaged marrow.
2. An insulin sensitizer drug, namely rosiglitazone in insulin-sensitizing dose had similar effects on normal and damaged bone marrow than insulin.
3. Rosiglitazone pre-treatment accelerated recovery of 5-fluorouracil-damaged bone marrow. Consequently neutropenia was milder.
4. G-CSF, the recently used agent supporting bone marrow regeneration, prior to chemotherapy actually may worsen the toxic effects on bone marrow. In contrast to these observations concurrent use of rosiglitazone with 5-FU repeatedly during 7 consecutive days did not unfavourably influence the population of the CFU-GM progenitor cells.
5. The accelerated recovery based upon a decreased susceptibility of CFU-GM progenitors to 5-FU-toxicity by the end of the 5-day rosiglitazone pre-treatment.
6. Myeloprotection was partly due to direct effects on progenitors, regarding similar effects were observed in the *in vitro* bone marrow cell cultures than *in vivo*.
7. *In vitro* rosiglitazone's beneficial effects were neutralized by a peroxisome-proliferator-activated receptor gamma (PPAR γ) antagonist.
8. Rosiglitazone's beneficial modifying effect was observed in the similar dose range on the human progenitor cells, than on the murine CFU-GM.

Significance

The major finding of the present Thesis is that rosiglitazone is able to confer protection on bone marrow cells against 5-FU-induced myelotoxicity. Our finding that a simple thiazolidinedione molecule may have a myeloprotective effect would be an importance in practice. Easily orally administration covers patient's desire and may improve compliance. Thiazolidinediones, rosiglitazone and pioglitazone, belong to the most frequently sold 100 pharmaceuticals in the world (Mosby 2003). Of course they have side effects, especially hepatic injury has to be controlled, but millions of people use them without remarkable adverse effects. Regarding that the presently used protective agents, hematopoietic stimulating factors and amifostine have uncomfortable side effects in large percentage of patients and they are administered parenterally subcutaneously or intravenously, the easy orally use of thiazolidinediones may be an advantage to improve the compliance of the patients. During chemo- and radiotherapy the least tolerable side effects for the patients are nausea and vomiting, which can even lead to refusal of therapy. Aggravating such effects would not be desirable. Amifostine results in nausea and vomiting in about the half of the patients. Rosiglitazone precipitates nausea less than the 0.5 percent of the patients.

Rosiglitazone may have many direct and indirect supportive effects on hematopoiesis, which may be important, especially considering that thiazolidinediones can inhibit many types of malignant cell lines including acute myeloid leukemic cells (Asou et al. 1999, Ohta et al., 2001, Fujimura et al., 1998; Mossner et al., 2002; Toyoda et al., 2002). Rosiglitazone may protect normal CFU-GM progenitor cells against toxicity of cytostatic agents. The myeloprotective effects of rosiglitazone may also prevent infections associated with neutropenia caused by chemotherapy, which is the highest cause of mortality in malignant diseases. Being able to avoid such disturbing side effects of chemotherapy as oral candidiasis and other chronic mycoses obviously helps to improve quality of life of the patients. It may also have an importance in pharmacological *ex vivo* purging of peripheral blood progenitor cell collections. Thus rosiglitazone might be a real alternative to the existing myeloprotective drugs in the future but further studies are warranted to evaluate the optimal treatment schedules.

Publications on which this Thesis is based on:

Original research articles:

1. Benkő, I., K. Djazayeri, C. Ábrahám, J. Zsuga, Z. Szilvássy: Rosiglitazone-induced protection against myelotoxicity produced by 5-fluorouracil. Eur. J. Pharmacol. 477, 179-182, 2003.
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2. Djazayeri, K., Z. Szilvássy, B. Peitl, J. Németh, L. Nagy, A. Kiss, B. Szabó, I. Benkő. Accelerated recovery of 5-fluorouracil-damaged bone marrow after rosiglitazone treatment. Eur J Pharmacol 522, 122-129, 2005.
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3. Djazayeri, K., Z. Szilvássy, K. Benkő, B. Rózsa, B. Szabó, A.J. Szentmiklósi, I. Benkő. Effect of rosiglitazone, an insulin sensitizer, on myelotoxicity caused by repeated doses of 5-fluorouracil. Pharmacol. Res. Közlésre elfogadva
IF: 0,740

Other publications

Abstracts

1. I Benkő, K Djazayeri, P Literati-Nagy, G Rablóczy, J Zsuga, B Szabó, Z Szilvássy: Insulin-sensitization as a novel chemoprotective mechanism. The Hematology Journal, 4, S2, 39, 2003.
2. Benkő, I., K. Djazayeri, B. Rózsa, Zs. Kovács, Z. Dinya, A.J. Szentmiklósi Protective effects of fruit extract with high polyphenol content against doxorubicin-induced myelotoxicity *in vivo*. Fund Clin Pharmacol, 18. S1, 89., 2004.
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Presentations

1. I Benkő, K Djazayeri, P Literati-Nagy, G Rablóczy, J Zsuga, B Szabó, Z Szilvássy: Insulin-sensitization as a novel chemoprotective mechanism. 8th Congress of European Hematology Association , Lyon, 2003. , France
2. K. Djazayeri, Z. Szilvássy, I. Benkő: Rosiglitazone, an insulin sensitizing drug, fastened regeneration of bone marrow damaged by 5-fluorouracil in mice. 4th Meeting of the Federation of European Pharmacological Societies, EPHAR, 2004, Porto, Portugal
3. Benkő, I., K. Djazayeri , B. Rózsa, Zs. Kovács, Z. Dinya, A.J. Szentmiklósi: Protective effects of fruit extract with high polyphenol content against doxorubicin-induced myelotoxicity in vivo. 4th Meeting of the Federation of European Pharmacological Societies, EPHAR, 2004, Porto, Portugal
4. B Rózsa, B Szabó, K Djazayeri, T Erdélyi, Zs Szoby, Z Dinya, J Szentmiklósi, I Benkő: Protective effects of fruit extract with high polyphenol content against doxorubicin-induced myelotoxicity in vivo. Magyar Molekuláris és Prediktív Epidemiológiai Társaság II. Nemzetközi Kongresszusa. Pécs, 2005.
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