Hypoxia Controls the Hemostasis of Naturally Occurring Regulatory T Cells via Hypoxia Inducible Factor-1

Jeremy Ben-Shoshan [1,2], Sofia Maysel-Auslender [1], Mor Adi [1,2], Gad Keren [1,2], Jacob George [1,2]

[1] Cardiology Department, Tel-Aviv Sourasky Medical Center, [2] "Sackler" School of Medicine, Tel-Aviv University, Tel Aviv, Israel

Background - Hypoxia is a negative regulator of T cells stimulation, cytokine production and proliferation, inducing a shift towards T_H2-cell responses and inhibition of T_H1-cell responses. Recently, different studies have reported the involvement of hypoxia inducible factor-1 (HIF-1) in the regulation of T cell proliferation and activation. Naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) are a subpopulation of suppressive lymphocytes which maintain immune homeostasis and self tolerance. Herein, we aim to investigate the effect of hypoxia and HIF-1α activation on the occurrence and function of Tregs.

Methods and Results **-** Incubation of Jurkat T cells under hypoxia ($1\%O_2$) resulted in a marked increase in both HIF-1α activity and Foxp3 expression as determined by ELISA and flow cytometry, respectively. This effect was abolished by retroviral transduction of siRNA directed against HIF-1α. Additionally, transduction of stabilized HIF-1α in Jurkat cells dose dependently increased Foxp3 expression levels. Exposure of human peripheral blood-derived mononuclear cells and mouse splenocytes to hypoxic conditions resulted in a significant increase in $Foxp3^+$ population among $CD4^+CD25^+$ cells. This was accompanied by an elevated capacity of hypoxia-treated $CD4^+CD25^+$ isolated cells to suppress the proliferation of $CD4^+CD25^-$ responders. Eventually, HIF-1α over-expression in mouse splenocytes by hydrodynamic naked DNA injection resulted in elevated mRNA levels of Foxp3 and total $CD4^+CD25^+Foxp3^+$cells number, compared to control.

Conclusions – Hypoxia regulates the hemostasis of naturally occurring regulatory T cells via expression of HIF machinery.

The Haptoglobin 2 Genotype is Associated with Increased Redox Active Hemoglobin Derived Iron in the Atherosclerotic Plaque

Shiri Kalet-Litman, Rabea Asleh, Rachel Miller-Lotan, Andrew P Levy

Lab of Vascular Medicine, Faculty of Medicine, Technion, Haifa, Israel

Background. The Haptoglobin (Hp) gene is polymorphic with two classes of alleles (1 and 2).The Hp 2-2 genotype is a major determinant of CV risk in DM individuals. We hypothesized that Hp genotype dependent differences in protecting against hemoglobin induced oxidative injury and in promoting clearance of hemoglobin would be manifested as increased oxidation from hemoglobin derived iron in the atherosclerotic plaque.

Methods and Results. Immunohistochemical analysis of plaques from C57Bl/6J Apo $E^{-/-}$ Hp 1-1 or C57Bl/6J Apo $E^{-/-}$ Hp 2-2 mice demonstrated a marked increase in plaque hemoglobin in Hp 2-2 mice. In order to determine if plaque hemoglobin could be derived from extravasation of hemoglobin we injected ^{125}I labeled Hp-Hb complexes into Apo $E^{-/-}$ mice and found markedly greater extravasation of the Hp 2-Hb complex into the plaque. Oxidation in the plaque was monitored by assessing in situ oxidation induced fluorescent activation of Dihydrorhodamine (DHR). DHR fluorescence was increased in Hp 2 plaques and was found to correlate and colocalize with iron and hemoglobin. The oxidation of DHR in the plaque was inhibited by iron chelation.

Conclusions. The increased oxidation present in Hp 2 plaques appears to be due to hemoglobin iron. Agents which prevent iron induced oxidation may have considerable value in decreasing oxidation and inflammation in Hp 2 plaques thereby reducing the risk of plaque rupture and atherothrombosis in Hp 2 DM individuals.

Effect of Pacing Lead Position on Ischemic Left-Ventricle Functioning: Should We Change the Strategy of Lead Placement in Cardiac Resynchronization Therapy?

Nitai Hanani [1], Rona Shofti [2], Eugene Konyukhov [1], Carmit Levi [1], Avshalom Shenhav [1], Amir Landesberg [1]

[1] Biomedical Engineering, [2] Medicine, Technion -IIT, Haifa, Israel

Background: Although the benefit of Cardiac Resynchronization Therapy is widely recognized many patients with ischemic cardiomyopathy fail to show improvement. We hypothesize that beneficial local myocardial functions will be obtained by pacing the weak ischemic regions, since pacing decreases energy consumption at the pacing site. The study tests the local and global short term effects of the opposing strategies: pacing at the lateral 'last activated' site or at the ischemic site. ***Methods:*** Myocardial infarction (MI) was created in the anteroseptal region by ligation of a large branch of the LAD, in open chest anesthetized sheep (n=5). A flowmeter was placed on the ascending aorta. Sonocrystals were implanted in the LV endocard and in the ischemic region. Local and global LV functions were assessed before and after the coronary ligation in three pacing modes: normal sinus, septal pacing at the ischemic site or lateral pacing. ***Results:*** At both baseline and ischemia, pacing on either site didn't significantly affect the global EW. However, huge decrease in the anteroseptal work was observed during local pacing ($p<.005$) while the lateral pacing slightly increases the work of the ischemic region. Moreover, pacing in the ischemic region diminished the post-systolic shortening work (active work during diastole) and thus improves the diastolic function ($n=3$, $p<.05$). ***Conclusions:*** Redistribution of the workload, reducing the work of weaker areas and loading the healthier regions, is feasible through pacing the ischemic regions. Improving the balance between mechanical demands and energy supply and improving cardiac diastolic function may promote myocardial reverse remodeling.

Toll-like Receptor 4 (TLR4) and Macrophage Migration Inhibitory Factor (MIF) Expression in the Myocardium Following Ischemic Injury or LPS Injection

Reout Fallach [1,2], Asher Shainberg [1], Yelena Chepurko [2], Eyal Porat [2], Michael Fainblot [2], Edith Hochhauser [2]

[1] *Faculty of Life Sciences, Cardiac Research, Bar-Ilan University, Ramat Gan,*
[2] *Cardiothoracic Surgery, The Cardiac Research Laboratory, Felsenstein Medical Research Center, Tel Aviv University, Rabin Medical Center, Petach Tikva, Israel*

MIF and TLR4 play an important role in the regulation of innate immune responses. Inflammation participates in the pathology of ischemic heart disease and in myocardial contractile depression during septic shock. The inflammation in cardiovascular disease is associated with the activation of immune cells and cardiac myocytes, which secrete interleukin 1 beta (IL-1 β) and tumor necrosis factor alpha (TNFα).
Aim: To investigate the time course of myocardial dysfunction and cytokine expression in models of LPS induced sepsis and myocardial ischemia (MI) induced by LAD ligation, and to determine the changes in the myocardial gene and protein expression of TLR4 and MIF.
Mice were challenged to sepsis with LPS or subjected to MI. Myocardial levels of IL-1β and TNFα increased significantly after MI or LPS injection reaching the maximum at 4 hours. The IL-1 β and TNF α levels remained high in the MI group, whereas in LPS injected group, levels returned to baseline within 72 hours. The decrease in hemodynamic function following LPS injection was transient, maximizing at 4 hours (78±11 mmHg, 65 % of baseline values). In MI hemodynamic function decreased to 67±8.5mmHg, 58 % of baseline values, after 24 hours ($p<0.05$), remaining so for 72 hours. Although myocardial TLR4 and MIF gene expression increased after LPS and MI challenge, the TLR4 protein expression remained unchanged while MIF increased after an initial decrease.
Conclusion: In addition to their role in the immune response, TLR4 and MIF depress cardiac function in both ischemia and LPS injury.

Rapamycin Protects Heart Cultures against Hypoxia via Ryanodine Receptors

Dalia El-Ani [1,3], Michael Arad [1,2], Tova Zinmann [3], Guy Kutz [1,2,3], Victor Guetta [1,2], Asher Shainberg [3]

[1] Heart Institute, Sheba Medical Center, Ramat Gan, [2] Sackler School of Medicine, Tel Aviv University, Tel-Aviv, [3] Faculty of Life Science, Bar-Ilan University, Ramat Gan, Israel

Introduction: Ryanodine receptors (RyR2), the major intracellular Ca^{2+} release channel in the cardiac muscle, play an essential role in excitation-contraction coupling by regulating the release of Ca^{2+} from the sarcoplasmic reticulum (SR) for binding to the contractile apparatus. RyR2 channel function is a subject to exquisite levels of modulation via diverse mechanisms, including interaction with accessory proteins such as FKBP12.6. Rapamycin (sirolimus) is an antibiotic that inhibits protein synthesis through mammalian target of rapamycin (mTOR) signaling and is used as an immunosuppressant. The proposed mechanism for the anti proliferative effect of rapamycin is based on its ability to bind to its intracellular receptor, the FK506 binding protein (FKBP12.6). Rapamycin confers preconditioning-like effect against myocardial infraction through opening of mitochondrial K_{ATP} channels. Our goal was to study the interactions between rapamycin and RyR2 in hypoxic heart cultures in order to elucidate the protective mechanism induced by rapamycin. Heart cultures were exposed to 90 min hypoxia and reoxygenation. Rapamycin (10 μM) and ryanodine (2 μM) attenuated by 40-50% LDH or CK leakage from cardiomyocytes that were subjected to hypoxia and reoxygenation. Ryanodine (100 μM, a concentration that closes the channels) chelerythrine (2 μM -PKC inhibitor) abolished the protective effect of rapamycin, indicating that opening of ryanodine channels are involved in the protective mechanism of rapamycin against hypoxia. Desmin immunostaining and MTT measurements confirmed the result of cardioprotection. In order to study the mechanism of cardioprotective effect of rapamycin, $[Ca^{2+}]_i$ was recorded in cells that were loaded with indo-1. Fluorescent ratio of 410/490 nm was measured. Rapamycin caused $[Ca^{2+}]_i$ elevation by about 20%, which was accompanied with inhibition of spontaneous contractility. This elevation of $[Ca^{2+}]_i$ and inhibition of contraction lasted as long as rapamycin remained in the culture dish. Caffeine caused the release of Ca^{2+} from the SR. However, if caffeine was given following rapamycin then it did not cause $[Ca^{2+}]_i$ release, probably because of SR depletion of Ca^{2+} by rapamycin. Pretreatment with ryanodine (100 μM), prevented rapamycin-induces $[Ca^{2+}]$ release. Conclusion: Rapamycin protects heart cultures against hypoxia via opening of ryanodine receptors, elevating of cytosolic $[Ca^{2+}]$ and activating PKC.

Bone Marrow from Exercise Trained Diabetic Rats Induces Angiogenesis in the Mice Ischemic Hind Limb

Merav Klo [1], Radka Holbova [2], Itzhak Binderman [3], Mickey Scheinowitz [4]

[1] Neufeld Cardiac Research Institute, Tel Aviv University, Tel Aviv, [2] Neufeld Cardiac Research Institute, Sheba Medical Center, Tel Hashomer, [3] Biomedical Engineering, [4] Neufeld Cardiac Research Institute, Biomedical Engineering, Tel Aviv University, Tel Aviv, Israel

The prevalence of cardiovascular diseases increases with age and moreover in diabetic patients and it is associated with reduced angiogenesis potency. **Purpose:** To investigate the capability of exercise in diabetic aged rats to induce angiogenesis in mice hind limb ischemia following bone marrow (BM) transplantation. **Methods:** One year old diabetic (Streprozotocine) and non diabetic SD male rats (n=22) were assigned to 2 weeks of exercise using voluntary activity wheel (Ex&Diab) or remained sedentary (Sed; Sed&Diab). There after, whole BM was aspirated and transplanted to the center of the hind limb ischemia (0.3x10^6 cells in 2 injections) of nude female mice 3 days after inducing the ischemia. Mice kept 2 weeks until sacrificed. Limb perfusion was measured with Laser Doppler on day 0, 7, and 14. Upon sacrifice, the muscle tissue was removed and stained with BS-1 lectin. **Results:** Aged exercise rats accumulated on average 164±100 meters/day while aged Ex&Diab rats accumulated 60±17 meters/day, p=0.03. Limb function showed an 80%±14% recovery in the Ex&Diab BM transplanted mice compared with a 88%±9.7% recovery in the Sed&Diab mice (p=NS). Capillary number was significantly higher in the Ex&Diab BM transplanted mice compared with their sedentary counterpart (p=0.0373) and it was correlated with improved tissue perfusion (51.1%±8.6% vs. 37.4%±5.7%, for Ex&Diab vs. Sed&Diab respectively, p=0.031). **Conclusions:** Exercise training stimulates BM of aged, diabetic rats which demonstrated angiogenic capacity after transplanted to hindlimb ischemia in mice. Our preliminary findings emphasize the role of exercise on bone marrow regeneration in advanced age and with diabetes.

The Molecular Mechanisms Responsible for the Divergent Regulation of Cytokine Secretion by Macrophages after Binding Hb-Hp1 and Hb-Hp 2 to CD163

Merav Strauss, Andrew P Levy

Lab of Vascular Medicine, Faculty of Medicine, Technion, Haifa, Israel

We have recently demonstrated that the haptoglobin 2-2 genotype is associated with an increased risk for diabetic cardiovascular disease. In addition to serving as an antioxidant it has been proposed that Haptoglobin, as mediated through the CD163 receptor, may also have an immunomodulatory function.

In this work, we sought to determine if the protein products of the two haptoglobin alleles differed in their ability to modulate the cytokine profile produced by macrophages in response to the haptoglobin-hemoglobin complex. Human PBMCs were cultured in the presence of complexes formed by the protein products with hemoglobin. We found that the haptoglobin 1-hemoglobin complex stimulated the secretion of significantly more Il-6 and Il-10 than the haptoglobin 2 -hemoglobin complex, and that the release of these cytokines is dependent on the binding of the haptoglobin-hemoglobin complex to the CD163 receptor.

Studies using specific kinase inhibitors have revealed that both CK2 and PKC are involved in the CD163 signaling mechanism that leads to increase in cytokine production. We found that there is no significant difference in the ratio of the amount of CD163/casein kinase 2ά or β associated with CD163 between cells that were or were not stimulated with Hp-Hb complex. However we found that binding of Hp1-1:Hb result in a significant increase in CK2 activity associated CD163 compare to Hp2-2:Hb. We conclude that Hp1-1:Hb increased cytokine production compare to Hp2-2:Hb due to increase in the activity of of CK2. We also showed that phosphorylation of CK2 is critical for its increase in specific activity.

Circulating Apoptotic Progenitor Cells in Congestive Heart Failure

Shmuel Schwartzenberg, Dael Geft, Ariel Finkelstein, Sofia Maysel-Auslender, Dov Wexler, Gad Keren, Jacob George

Cardiology, Tel Aviv Medical Center, Tel Aviv, Israel

Background: Circulating CD34+ endothelial progenitor cells (EPCs) are increased in conditions associated with ischemia and can potentially support angiogenesis and vasculogenesis. EPC levels were found to correlate positively with NYHA level and all-cause mortality in congestive heart failure (CHF). Recently, we identified a novel population of apoptotic progenitor cells which was elevated in patients with acute coronary syndrome (ACS).

Aims: we sought to determine whether apoptotic progenitor cells are elevated in patients with heart failure, similarly to ACS patients. In so doing, we considered early, reversible apoptotic CD34 cells and late, irreversible apoptotic progenitors, whose plasma membrane is no longer intact and thus represent necrotic cells.

Methods: Peripheral blood mononuclear cells were isolated by Ficoll density-gradient from 58 patients with various degrees of heart failure and 26 healthy controls. Apoptosis in progenitor CD34+ cells was assessed using the Annexin V-PE/PI detection kit, and FACS analysis was performed with triple staining for CD34, annexin-V and propidium iodide. The percentage of early and late apoptotic progenitor cells was determined in the subject groups and was correlated with clinical characteristics.

Results: There was no significant difference in total CD34+ cells or early apoptotic progenitors between healthy subjects and CHF patients ($p=0.326$) or between severe vs mild/moderate CHF groups ($p=0.544$). We found an elevated number of late apoptotic progenitors in the severe CHF group compared with the mild/moderate CHF group ($p= 0.03$). There was also an inverse correlation between late apoptotic progenitors and ejection fraction ($r = -0.252$, $p=0.028$) as well as a positive association with NYHA class ($r = 0.223$, $p=0.046$).

Conclusions: In the interplay between cytokines enhancing progenitor cell mobilization and those precipitating their apoptosis, our results could support the hypothesis that increasing severity of heart failure shifts the balance towards enhanced progenitor cell apoptosis. The lower the ejection fraction, the poorer the forward flow, which may increase tissue ischemia and endothelial damage. Therefore, apoptotic progenitor cells ought to be evaluated in future studies as a potential predictive biomarker in CHF.

Altered Hemostasis of Naturally Occurring Regulatory T Cells in Patients with Congestive Heart Failure.

Arnon Adler [3], Sophia Maysel-Auslender [3], Dov Wexler [1], David Sheps [1], Gad Keren [3], George Jacob [2]

[1] *Cardiology, Heart Failure Clinic,* [2] *Cardiology, Cardiac Catheterization Unit,*
[3] *Cardiology, Tel-Aviv Souraski Medical Center, Tel Aviv, Israel*

Background: Patients with congestive heart failure (CHF) have been shown to exhibit dysregulation of the immune system. Naturally occurring CD4+CD25+Foxp3+ regulatory T cells (Tregs) are a subpopulation of lymphocytes which are thought to play a significant role in regulation of the immune system. Their number and function is compromised in certain disorders involving the immune system such as SLE and MS and in acute coronary syndrome. We hypothesized that the number of Tregs in patients with CHF will differ from that of healthy subjects.

Methods: Peripheral blood mononuclear cells were isolated using Ficoll density gradient and triple-stained with antibodies to CD4, CD25 and Foxp3. The number of Tregs was evaluated by a Fluorescence activated cell sorter (FACS).

Results: 55 patients with CHF, including 46 with coronary artery disease, and 28 healthy subjects were examined. The number of CD4+CD25+Foxp3+ cells was significantly higher in the CHF group in comparison with the control group (3.07% ± 1.04 & 1.71% ± 0.56 respectively, age matched P value= 0.002). Subgroup analysis showed that Treg numbers in patients with CAD and CHF were significantly higher in comparison with non-ischemic CHF patients (3.26% ± 0.97 & 2.12% ± 0.84 respectively, age matched P value= 0.019).

Conclusions: In patients with CHF the number of CD4+CD25+Foxp3+ Tregs is increased. This may further our understanding of the involvement of the immune system in CHF.