Co-administration of Adenoviral VEGF and Ang-1 Enhances Vascularization and Reduces Ventricular Remodeling in the Infarcted Myocardium of Type I Diabetic Rats

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Running Title: Rescue of diabetic myocardium by VEGF/Ang-1 Therapy

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Objective: - Hyperglycemia impairs angiogenesis in response to ischemia, leading to ventricular remodeling. Although the effects of overexpressing angiogenic growth factors have been studied in inducing angiogenesis, the formation of functional vessels remains a challenge. The present study evaluates the reversal of diabetes mediated impairment of angiogenesis in the infarcted diabetic rat myocardium by pro-angiogenic gene therapy.

Research Design and Methods: - Ad.VEGF and Ad.Ang1 were intramyocardially administered in combination immediately after myocardial infarction to non-diabetic and diabetic rats. Ad.LacZ was similarly administered to the respective control groups. The hearts were excised for molecular and immunohistochemical analysis at predetermined time points. The myocardial function was measured by echocardiography 30 days after the intervention.

Results: - We observed reduced fibrosis and increased capillary/arteriolar density along with reduced ventricular remodeling, as assessed by echocardiography in the treated diabetic animals when compared to the non-treated diabetic controls. We have also observed increased p-MK2, 2 days after the treatment and increased expression of VEGF, Flk-1, Ang-1, Tie-2, and survivin, 4 days after treatment in the diabetic animals. Gel shift analysis revealed that the combination gene therapy stimulated the DNA binding activity of NFκB in the diabetic animals.

Conclusions: - Our preclinical data demonstrates the efficacy of co-administration of adenoviral VEGF and Ang-1 in increasing angiogenesis and reducing ventricular remodeling in the infarcted diabetic myocardium. These unique results calls for the initiation of a clinical trial to assess the efficacy of this therapeutic strategy in the treatment of diabetes related human heart failure.
Diabetic individuals who develop an Ischemic Heart Disease (IHD) sustain an unfavorable prognosis for survival than other IHD subjects without diabetes (1). This condition may be attributed to the impaired coronary collateral vessel development and reduced myocardial vascular perfusion in response to ischemia leading to profound ventricular remodeling and subsequent heart failure (2). Various studies have linked diabetes mediated impaired myocardial angiogenesis to alterations in the delicate balance of angiogenic growth factors and cytokines regulating vascular stability (2-4) and compromised signal transduction (4). Several studies have reported the possible role of decreased Vascular Endothelial Growth Factor (VEGF) and Angiopoietin-1 (Ang-1) in the pathogenesis of diabetes mediated impairment of angiogenesis in the myocardium (5-7).

There have been several attempts at the preclinical and clinical levels to induce angiogenesis by overexpressing the angiogenic factors in the peri-infarct zone after Myocardial Infarction (MI). Most of the studies have approached this issue using a single gene as the therapeutic agent. Delivery of vectors encoding VEGF165 (VEGF) and VEGF-2 was shown to improve collateral vascular perfusion and nourish the oxygen depleted myocardium thereby reducing angina and improving the heart function in human clinical trials (8-10). However, investigations into the long term effects of sustained expression of VEGF in mice models revealed deleterious effects due to the formation of leaky immature vessels/hemangiomas and subsequent death of the experimental animal (11; 12). Furthermore, transgenic mice overexpressing VEGF revealed lengthy and leaky dermal vessels with evident inflammation (13; 14).

On the other hand, Ang-1 system is known to play a critical role in vascular maturation and stabilization thereby supporting VEGF induced neovascularization in a complementary manner (6; 14; 15). Recently Ang-1 gene therapy has been shown to support the maturation of the immature vasculature in db/db mice (16).

In recent past, work has been done to elucidate the synergistic effect of co-administration of VEGF and Ang-1 in ischemic rat myocardium (17-19). Zhou L, et al have reported that combined gene therapy using VEGF and Ang-1 significantly reduced myocardial infarct size through the induction of the PI-3 kinase and Bcl-2 survival pathways and Nuclear Factor-kappa B (NFκB) activation (18).

The prospect of a gene therapy using a combination of VEGF and Ang-1 encoding vectors to activate the angiogenic signaling cascade has not yet been explored in the diabetic ischemic myocardium. Diabetes reflects a far more challenging condition, where the VEGF and Ang-1 system is significantly downregulated, hampering the ability of the myocardium to respond to an ischemic stress (2; 6), and where the usual revascularization techniques such as Coronary Artery Bypass Graft (CABG) and Percutaneous Transluminal Coronary Angioplasty (PTCA) tend to fail thereby leaving many of the diabetic-IHD subjects with no option. Therefore, in this study we aimed at employing a combination gene therapy approach involving in vivo adenoviral gene delivery of VEGF and Ang-1, to enhance neoangiogenesis by the repairing the impaired angiogenic signaling cascade and thereby reduce ventricular remodeling in streptozotocin (STZ) induced Type I diabetic rats. Our findings emphasize the efficacy of co-administration of adenoviral vectors encoding VEGF and Ang-1 in inducing and stabilizing the process of angiogenesis that is relegated in the diabetic myocardium and reducing ventricular remodeling in the
infarcted myocardium in a diabetic milieu, thereby supporting the development of a combination gene therapy for therapeutic myocardial angiogenesis.

METHODS

**Experimental Animals:** This study was performed in accordance with the principles of laboratory animal care formulated by the National Society for Medical Research and with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (Publication No. 85-23, revised 1985). The experimental protocol was approved by the Institutional Animal Care Committee of the University of Connecticut Health Center (Farmington, CT). Male SD rats (300-325gm) were randomly separated into normal and diabetic rats as they received an (i.p.) injection of vehicle (0.1mol/l citrate buffer, pH 4.5) alone or streptozotocin (STZ) at a dosage of 65mg/kg body weight dissolved in 0.1mol/l citrate buffer.

**Experimental Design/Surgical Procedure:** MI was induced in the diabetic animals 30 days after the induction of diabetes as previously described (20). Age matched non-diabetic animals were used as comparable controls. The rats were randomized into 6 groups. 1) Control (non-diabetic) Sham (CS), 2) Diabetic Sham (DS) 3) Control (non-diabetic) MI (CMI) + Ad.LacZ (CLZMI), 4) CMI + {Ad.VEGF+Ad.Ang1} (CVAMI), 5) Diabetic MI (DMI) + Ad.LacZ (DLZMI) and 6) DMI + {Ad.VEGF+Ad.Ang1} (DVAMI). Ad.VEGF and Ad.Ang1 were generous gifts from Dr. Li C, East Tennessee State University, Tennessee, USA (18). Dr. Li C’s groups have verified the transfection efficiency of these adenoviral vectors in vitro. Moreover the authors have demonstrated the synergistic effect of co-administration of VEGF and Ang1 in the ischemic rat myocardium (18). Therefore in our current investigation the adenoviral dosage was used based on Zhou et al’s observations (18).

Immediately after MI, a mixture of adenoviral vectors encoding for VEGF (Ad.VEGF, 6 x 10⁷ pfu) and Ang-1 (Ad.Ang1, 1.5 x 10⁵ pfu) were intramyocardially administered in combination (in 100µl of PBS, using a 30g needle) at 4 sites at the border zone of the ischemic area in the CVAMI and DVAMI groups. Adeno-LacZ (Ad.LacZ, 6x10⁷ pfu) was used as the control adenoviral vector in the CLZMI and DLZMI groups, in order to nullify any possible effects exerted by the vector itself.

For detailed descriptions of the Materials and Methods please refer to the supplementary material available in the online appendix at http://diabetes.diabetesjournals.org.

**Statistical Analysis:** All data were analyzed by statistical software ‘GraphPad Prism PC software’ (SanDiego, CA, USA) version 5.0. Statistical analysis was performed by using one way analysis of variance (ANOVA). Post-hoc comparisons between the groups were performed by Newman-Keuls Multiple Comparison Test. Results are presented as mean ± SEM with p ≤ 0.05 used to indicate statistical significance.

RESULTS

The result demonstrating the efficacy of intra-myocardial gene delivery (Fig. 1A) has been included in the supplemental file in the online appendix.

**Immunohistochemical analysis of VEGF and Ang-1.** There was a decrease in the expression of VEGF and Ang-1 in the diabetic Sham (DS) when compared to the non-diabetic control (CS) (Fig. 1B and 1C). The MI induced by LAD occlusion seemed to trigger the expression of VEGF and Ang-1 in the non-diabetic control (CLZMI) animals to which Ad.LacZ was administered. However, this infarction triggered expression of the
angiogenic growth factors seemed to be impaired in the diabetic animals (DLZMI, Fig. 1B and 1C). The co-administration of Ad.VEGF and Ad.Ang1 in the non-diabetic (CVAMI) group markedly induced expression of both these growth factors. Similarly the expression of VEGF and Ang-1 was restored markedly upon therapy treatment in the diabetic (DVAMI) myocardium (Fig. 1B and 1C).

**Biological Effects of Co-expression of VEGF and Ang-1 in the Diabetic Ischemic Myocardium—Myocardial Fibrosis (Masson’s Trichrome Staining).** To determine the effect of the combination gene therapy on the extent of myocardial fibrosis, Masson’s trichrome staining was performed on paraffin embedded heart tissue sections 7 and 30 days after the surgical intervention. After 7 days of MI, there was significant increase in the myocardial fibrosis in the DLZMI group (30.8 ± 2.8%) compared to the CLZMI (23.9 ± 0.5%) group. Upon therapy the non-diabetic CVAMI (20.2 ± 0.4%) group demonstrated reduced collagen deposition and fibrosis when compared with the respective Ad.LacZ treated non-diabetic CLZMI (23.9 ± 0.5%) group. Similarly, the diabetic MI group (DVAMI, 20.1 ± 0.7%) that received the therapy demonstrated significantly reduced myocardial collagen deposition and fibrosis when compared with the respective Ad.LacZ treated diabetic MI group (DLZMI, 30.8 ± 2.8%) (Fig. 2A). Fig. 2A shows heart tissue sections of the diabetic MI animals (DVAMI) that received the therapy with less fibrosis when compared with the Ad.LacZ treated diabetic MI group (DLZMI) which showed increased fibrosis and ventricular dilatation. CS and DS (Fig. 2A and 2B) represents the non-diabetic and diabetic Sham operated groups with no significant fibrosis. We have observed a similar trend after 30 days of intramyocardial gene therapy (Fig. 2B). The diabetic MI group (DVAMI, 16.5 ± 1.9%) that received the therapy showed reduced myocardial collagen fibrosis when compared with the respective Ad.LacZ treated diabetic MI group (DLZMI, 20.1 ± 1.1%). Evidently there was a less prominent scar extension in the treated diabetic rats (DVAMI) compared to the Ad.LacZ treated (DLZMI) animals (Fig. 2B).

**Capillary Density and Arteriolar Density.** In order to understand whether the reduction in fibrosis was paralleled by neoangiogenesis, we performed immunohistochemical analysis for CD31 (endothelial cell marker) after 7 days of the therapy which allowed estimation of the density of CD31 positive vessels (capillary density in counts/mm²) (Fig.3A and 3B). There was a significant decrease in the capillary density in the diabetic Sham operated (DS, 2706 ± 122) and Ad.LacZ treated diabetic MI groups (DLZMI, 1769 ± 12) when compared to the non-diabetic Sham (CS, 3619 ± 214) operated and Ad.LacZ treated non-diabetic MI (CLZMI, 2308 ± 130) groups, respectively. In the present study we have observed an increase in the myocardial capillary vessel density in the diabetic MI animals (DVAMI, 2206 ± 159) which received the adenoviral vectors encoding VEGF and Ang-1 in combination when compared to the Ad.LacZ treated diabetic MI (DLZMI, 1769 ± 12) animals. Similar results were observed in non-diabetic MI (CVAMI) group which received the therapy (2853 ± 123) when compared to the Ad.LacZ treated non-diabetic MI (CLZMI, 2308 ± 130) group.

In order to analyze the angiogenic response to the therapy the arteriolar density (in counts/mm²) was measured by immunostaining the heart tissue sections (Fig. 3C and 3D) after 7 days of gene therapy for α-SMA (alpha smooth muscle actin). There was a decrease in the arteriolar density in the diabetic Sham operated (DS, 15.8 ± 1.8) and Ad.LacZ treated diabetic MI groups (DLZMI, 16.3 ± 2.9) when compared to the non-diabetic Sham (CS, 28.3 ± 1.3) operated and
Ad.LacZ treated non-diabetic MI (CLZMI, 29.2 ± 5.5) groups, respectively. In the present study we have observed a significant increase in the number of arterioles (arteriolar density) in the diabetic MI (DVAMI) animals which received the therapy (31.3 ± 0.9) when compared to the Ad.LacZ treated diabetic MI (DLZMI, 16.3 ± 2.9) group. Similar results were observed in non-diabetic MI group (CVAMI) which received the therapy (50.2 ± 3.9) when compared to the Ad.LacZ treated non-diabetic MI (CLZMI, 29.2 ± 5.5) group.

Molecular Basis for the Angiogenic and Cardioprotective Effect of VEGF-Ang-1 Therapy in the Diabetic Ischemic Myocardium—Effect of gene therapy on the expression of VEGF and Flk-1. We have observed a significant reduction in the expression of VEGF (1.75 fold) and Flk-1 (7.1 fold) 4 days after the surgical procedure in the Sham operated diabetic animals (DS) when compared to the Sham operated non-diabetic control (CS) (Fig. 4A and 4B). Similarly, there was a significant reduction in the expression of VEGF (2.5 fold) and Flk-1 (2.5 fold) in the DLZMI group compared to the CLZMI group (Fig. 4C and 4D). However, there was a significant increase (1.6 fold) in VEGF expression in the non-diabetic Ad.LacZ treated MI (CLZMI) group when compared to the non-diabetic Sham operated controls (CS). This MI induced increase in the expression of VEGF was compromised in the diabetic MI (DLZMI) group that was treated with Ad.LacZ. Upon treatment with a combination of adenoviral vectors encoding VEGF and Ang-1 in the non-diabetic MI animals (CVAMI) we have documented an increase in the expression of VEGF (1.3 fold) and Flk-1 (1.3 fold) 4 days after the gene transfer when compared to the Ad.LacZ treated non-diabetic MI (CLZMI) group (Fig. 4C and 4D). Similarly, we have observed a significant increase in the expression of VEGF (4.0 fold) and Flk-1 (2.1 fold) in the diabetic animals that received the therapy (DVAMI), 4 days after the treatment when compared to the Ad.LacZ treated diabetic MI (DLZMI) group (Fig. 4C and 4D).

Effect of gene therapy on the expression of Tie-2. In the present study we have observed a significant reduction in the expression of Ang-1 (2.2 fold) and Tie-2 (3.1 fold) 4 days after the surgical procedure, in the Sham operated diabetic animals (DS) when compared to the Sham operated non-diabetic control (CS) (Fig. 4A and 4B). Upon treatment with a combination of adenoviral vectors encoding VEGF and Ang-1 in the non-diabetic MI animals (CVAMI) we have documented an increase in the expression of Ang-1 (1.3 fold) and Tie-2 (1.3 fold) 4 days after the gene transfer when compared to the Ad.LacZ treated non-diabetic MI (CLZMI) group. Similarly, we have observed a significant increase in the expression of Ang-1 (4.0 fold) and Tie-2 (2.1 fold) in the diabetic animals that received the therapy (DVAMI), 4 days after the treatment when compared to the Ad.LacZ treated diabetic MI (DLZMI) group (Fig. 4C and 4D).

Effect of gene therapy on the expression of surviving. There was a marked decrease in the expression of anti-apoptotic protein survivin in the diabetic Sham (DS, 2.0 fold) and diabetic Ad.LacZ (DLZMI, 2.3 fold) treated MI groups when compared to the non-diabetic Sham operated (CS) and Ad.LacZ treated non-diabetic MI (CLZMI) groups, respectively (Fig. 4A and 4B). We have observed a significant increase in the expression of survivin in the non-diabetic animals that received the therapy (CVAMI, 3.7 fold) 4 days after the treatment when compared to the Ad.LacZ treated non-diabetic MI (CLZMI) group (Fig. 4C and 4D). Similarly, we have observed a significant increase in the expression of survivin in the diabetic animals that received the therapy (DVAMI, 7.2 fold) 4 days after the treatment...
when compared to the Ad.LacZ treated diabetic MI (DLZMI) group (Fig. 4C and 4D).

**Effect of gene therapy on the phosphorylation of MK2 (p-MK2).** There was a marked decrease in the phosphorylation of Mitogen Activated Protein Kinase –Activated Protein Kinase-2 (MK2, at Threonine 334) in the diabetic sham (DS, 1.7 fold) and diabetic Ad.LacZ (DLZMI, 3.8 fold) treated MI groups when compared to the non-diabetic Sham (CS) operated and Ad.LacZ treated non-diabetic MI (CLZMI) groups, respectively 2 days after the surgical procedure (Fig. 5A-5D). We have observed a significant increase in the phosphorylation of MK2, 2 days after the treatment in the non-diabetic animals that received the therapy (CV AMI, 1.3 fold) when compared to the Ad.LacZ treated non-diabetic MI (CLZMI) group (Fig. 5C and 5D). Similarly, we have observed a significant increase in the phosphorylation of MK2, 2 days after the treatment in the diabetic animals that received the therapy (DV AMI, 3.9 fold) when compared to the Ad.LacZ treated diabetic MI (DLZMI) group (Fig. 5C and 5D).

Similarly the therapy significantly increased the levels of p-MK2 4 days after intervention in both the DVAMI and CVAMI groups when compared to the DLZMI and CLZMI groups, respectively. However, there was an evident decrease in the levels of p-MK2 in all the groups 4 days after the intervention when compared to 2 days after the intervention.

**Effect of gene therapy on the DNA binding activity of NFκB.** Gel shift analysis 2 days after the surgical procedure revealed significant decrease in the DNA binding activity of NFκB in the diabetic sham (DS) animals compared to the non-diabetic control sham (CS) animals (Fig. 5E). NFκB DNA binding activity was significantly elevated in the non-diabetic control animals that received the therapy (CVAMI) compared to the Ad.LacZ treated non-diabetic MI (CLZMI) group (Fig. 5E). Similarly, the DNA binding activity of NFκB was significantly restored upon the co-administration of adenoviral VEGF and Ang1 in the diabetic animals (DVAMI) when compared to their respective Ad.LacZ treated diabetic controls (DLZMI) (Fig. 5E).

**Preservation of myocardial functions after MI in the diabetic myocardium through VEGF/Ang-1 combination gene therapy.** In order to evaluate the in vivo functional consequences of intramyocardial combination gene therapy we performed echocardiographic analysis on the experimental animals in the different groups 30 days after the gene transfer. Anterior wall thickness showed a decrease in all the groups subjected to MI when compared to the Sham group; and the thinning of the anterior wall is more in the DLZMI group as compared with the diabetic group treated with VEGF and Ang-1 combination gene therapy (data not shown). Larger LV dimensions at systole were also detected in diabetic sham (5.8 ± 0.1) group as compared to non diabetic sham (4.5 ± 0.1) groups. Four weeks after LAD ligation the LVIDs (mm) was significantly larger in DLZMI rats (7.4 ± 0.3) as compared to non-diabetic CLZMI (6.5 ± 0.2) rats (Fig. 6A and 6B). However this increase in LV dimensions was found to be prevented in the DVAMI (6.5 ± 0.3) groups compared to DLZMI (7.4 ± 0.3) groups. Also non-diabetic CVAMI group (5.9 ± 0.1) showed better improvement compared to non-diabetic CLZMI (6.5 ± 0.3) groups (Fig. 6A and 6B). Along with the LV dimensions we also noticed better systolic functions measured by Ejection Fraction (EF in %) and Fractional Shortening (FS in %) in all the control groups as compared to their respective diabetic groups. We have observed a significant increase in the EF (%) in the combination gene therapy treated non-diabetic MI (CVAMI) group (57.3 ± 0.8 vs. 50.1 ± 2) and diabetic MI (DVAMI) group.
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(53.9 ± 2.2 vs. 43.8 ± 2) when compared to their respective Ad.LacZ treated non-diabetic (CLZMI) and diabetic (DLZMI) groups (Fig. 6C). Similarly there was significant increase in the FS (%) in the combination gene therapy treated non-diabetic MI (CVAMI) group (31.2 ± 0.5 vs. 26.6 ± 1.2) and diabetic MI (DVAMI) group (29.3 ± 1.5 vs. 21.6 ± 1.1) when compared to their respective Ad.LacZ treated non-diabetic (CLZMI) and diabetic (DLZMI) groups (Fig. 6D). There were evident signs of bradycardia in all the diabetic groups as compared to their respective control groups. In the present study we have observed improvement in the heart rate (HR, in beats/minute) in the combination gene therapy treated diabetic MI (DVAMI) group (239.2 ± 8.5) when compared to the Ad.LacZ treated diabetic (DLZMI) group (201.8 ± 5.4) (Fig. 6E).

DISCUSSION

The angiogenic growth factors are downregulated in the diabetic myocardium which in turn hampers the myocardial collateral vessel formation as an adaptation to ischemia (2). Therefore, supporting the overexpression of these factors by means of gene therapy might aid in repairing the process of impaired angiogenesis in the diabetic ischemic myocardium. However, therapy using vectors encoding a single angiogenic factor has shown less significant improvement than what was expected, mainly because the biological system requires a cascade of growth factors and responsive intracellular signaling mechanisms for the development of a fully functional vascular system (21-23). Therefore, therapeutic angiogenesis is currently targeting combinations of angiogenic molecules as a therapeutic measure to induce myocardial angiogenesis (21). Ang-1 is known to modify VEGF responses to neovascularization by positively affecting vessel maturation and stability (24). A strategy to locally overexpress VEGF and Ang-1 in combination would prove to be beneficial because while VEGF can take the lead in the process of neovascularization, Ang-1 would be expected to support the maturation of the newly formed vessels.

We have observed significant reduction in the expression of VEGF and Ang-1 in the diabetic myocardium. However, MI induced the expression of VEGF in the Ad.LacZ treated non-diabetic MI group. This increase in VEGF in the non-diabetic MI group can be explained as an early adaptive response to the myocardial ischemia caused by LAD ligation (25; 26). This response was not seen in the diabetic MI animals confirming the impairment of the angiogenic machinery in the diabetic myocardium (27). In our present study, the strategy of combination gene therapy markedly increased the expression of VEGF and Ang-1 in the diabetic ischemic heart when compared to the Ad.LacZ treated diabetic MI group.

The impairment of myocardial angiogenesis in a diabetic condition has also been associated with compromised signaling mechanisms through the receptors, Flk-1 for VEGF and Tie-2 for Ang-1 (6; 27; 28). It is also known that the expression of these receptors is regulated by a positive feedback signaling mechanism where the ligand itself controls the levels of expression of its own receptors (6; 29). The downregulation in the expression of the receptors that we have observed in the diabetic myocardium corresponds to the impaired angiogenic signaling mechanisms associated with a diabetic milieu. The increase in the expression of the receptors Flk-1 and Tie-2 that we have observed upon gene therapy can be correlated to the increased expression of its ligands VEGF and Ang-1, respectively. Therefore the therapy might have been effective in correcting the angiogenic machinery directly associated with VEGF and Ang-1, which is impaired in the diabetic myocardium.
The VEGF/Flk-1 induced effects on endothelial cell migration through p38MAPK activation are primarily mediated by phosphorylation and activation of the p38MAPK substrate MK2 (30; 31). It was also reported that MK2 activation plays an essential role in VEGF induced actin reorganization, migration, and tubule formation in endothelial cells (32). Recently we have shown that Ischemic Preconditioning (IP) induces VEGF expression followed by Flk-1 activation thereby activating an angiogenic signaling cascade by the activation of p38MAPK and MK2 leading to the activation of NFκB (33). To elucidate the mechanism we used a heterozygous Flk-1 +/- and homozygous MK2-/- knockout mice. IP in the Flk-1 +/- failed to bring about significant phosphorylation of MK2 and increase DNA binding activity of NFκB when compared to the wild type mice myocardium subjected to IP. Similarly, when we studied the effects of ischemic preconditioning in MK2-/- knockout mice we have observed that the IP mediated activation of NFκB and the effective angiogenic response was significantly impaired when MK2 was knocked down (33). Thus we were able to confirm that downstream of Flk-1, activation of MK2 plays a crucial role in NFκB activation and angiogenesis in the myocardium. Our treatment strategy significantly increased the phosphorylation of MK2, which can be associated with the increase in the expression of VEGF and its receptor Flk-1 thereby triggering the angiogenic signaling pathway in the diabetic ischemic myocardium.

We have previously documented the effect of hypoxic preconditioning on NFκB activation and the role of NFκB in myocardial angiogenesis (34; 35). Both VEGF and Ang-1 has been shown to increase the DNA binding activity of NFκB thereby modulating endothelial cell proliferation, survival and migration (30). NFκB has been shown to activate several pro-survival genes, cytokines and Inhibitors of Apoptosis (IAP) necessary for endothelial cell survival (36). It was recently shown that combined gene therapy using VEGF165 and Ang-1 significantly reduced myocardial infarct size through the induction of NFκB activation (18). Our treatment strategy has significantly improved the DNA binding activity of NFκB in the diabetic ischemic myocardium thereby confirming improvement in the angiogenic signaling mechanism associated with increase in the expression of VEGF and Ang-1 and phosphorylation of MK2.

Survivin (an IAP) is upregulated by VEGF and Ang-1 in the endothelial cells thereby decreasing apoptosis (37-40). It has been reported that activation of NFκB signaling contributes to endothelial cell survival and tubular morphogenesis by the transactivation and upregulation of the pro-survival protein survivin (41). The reduced expression of survivin in the diabetic myocardium can be correlated to the increased myocardial fibrosis as evidenced by trichrome staining. Our treatment strategy leading to the co-expression of VEGF and Ang-1 might have led to the increased expression of survivin through the activation of NFκB signaling in the diabetic ischemic myocardium in the present study.

The co-operative effect of VEGF and Ang-1 co-expression and the increase in the expression of their respective receptors due to the therapy in mediating neovascularization was confirmed by a marked increase in the capillary density and arteriolar density in the DVAMI group when compared to the Ad.LacZ treated diabetic MI group. This increase in vascularization as a result of the therapy might have led to the significant reduction in the myocardial fibrosis and marked increase in the islands of viable cardiac tissue in the infarct and peri-infarct regions of the diabetic infarcted heart upon therapy. Although stabilization of the VEGF induced neo-vascularization by Ang-1 is the
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The most likely reason for the observed increase in capillary and arteriolar density in the surviving myocardium immediately adjacent to the infarct, the possibility of VEGF and Ang-1 induced cell survival, preservation of existing vessels and a resultant direct effect on myocardial repair cannot be ruled out. Moreover, there are evidences that suggest that post-MI vascular repair is not solely dependent upon the activation and participation of resident endothelial cells, but may also reflect an increased mobilization and homing of bone marrow derived endothelial progenitor cells (EPC) which is in turn activated by overexpression of both VEGF and Ang-1(42; 43). Therefore there is a possibility that our combination gene therapy might have induced EPC homing to the diabetic infarcted myocardium aiding in the reparative process.

A number of preclinical and clinical studies have shown structural changes in parallel with the functional changes of diabetic heart disease (44). Following MI, the surviving myocardium of non-diabetic subjects exhibits hyperkinesia in order to compensate for the infarcted myocardium in an attempt to maintain cardiac output. When it comes to a diabetic subject, in addition to the already compromised cardiac functions, after an MI, the myocardium is unable to achieve this compensatory enhancement in function due significant ventricular remodeling, myocardial fibrosis and collagen deposition, which in turn further reduces myocardial functional capability (44). In our current study, the reduced fibrosis and improved vascularization as a result of the therapy might have caused the improvement in the myocardial functions 30 days after MI in the diabetic rats. The therapy resulted in preservation of myocardial thickness and less prominent collagen deposition. Consistent with other reports, though bradycardia was evident in all the diabetic groups, the heart rate significantly improved upon treatment (45). The treatment resulted in better contractile function and suppression of the progressive cardiac failure that is associated with ventricular remodeling in a diabetic infarcted heart.

To the best of our knowledge our results have documented for the first time that intramyocardial coadministration of adenoviral vectors encoding VEGF and Ang-1 induces angiogenesis and vessel maturation thereby rendering cardioprotection against the ischemic stress induced by MI in STZ induced Type I diabetic rats. The therapy significantly reduced the ventricular remodeling as evidenced by the significant reduction in the collagenous fibrotic tissue and improvement in the myocardial functions in conjunction with significant increase in the levels of VEGF and its receptor Flk-1, Ang-1 and its receptor Tie-2, p-MK2 and anti-apoptotic survivin. Our unique and promising preclinical findings therefore support the development of a combination gene therapy for therapeutic myocardial angiogenesis and calls for the initiation of a clinical trial to assess the efficacy of this unique therapeutic strategy in the treatment of diabetes related human heart failure.

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REFERENCES


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Figure Legends

Figure 1: - (A) Representative micrographs showing the in vivo transfection efficiency of Ad.LacZ in the non-diabetic SHAM operated groups. Robust infection of the myocardium as assessed by β-gal staining in the viable cardiac muscle surrounding the sites of gene transfer can be seen in the Ad.LacZ transfected myocardium. (B) Expression of VEGF as assessed by immunohistochemical staining. (C) Expression of Ang-1 as assessed by immunohistochemical staining. The decrease in the expression of VEGF and Ang-1 is evident in the diabetic DS and DLZMI group when compared to the respective non-diabetic CS and CLZMI group. Increase in the expression of VEGF and Ang-1 can be seen in the groups that received the combination gene therapy (CV AMI and DV AMI) when compared to their respective (CLZMI and DLZMI) Ad.LacZ treated groups. (-) represents representative micrographs showing the sections in which primary antibody was not added to verify the specificity of the staining protocol. CS represents non-diabetic Control Sham, DS represents Diabetic Sham, CLZMI represents non-diabetic Control animals that received Ad.LacZ injections, DLZMI represents Diabetic animals that received Ad.LacZ injections, CVAMI represents non-diabetic Control animals that received combination gene therapy, DVAMI represents Diabetic animals that received combination gene therapy.

Figure 2: Effect of combination gene therapy on myocardial fibrosis 7 and 30 days after gene therapy. (A) Representative images show Myocardial Infarct and Fibrosis after 7 days of MI and combination gene therapy. (B) Representative images show Myocardial Infarct and Fibrosis after 30 days of MI and combination gene therapy. There is no evident fibrosis in the CS and DS groups. A thinner infarct and significant fibrosis is evident in the CLZMI and DLZMI groups. Combination gene therapy in the CVAMI and DVAMI groups resulted in a thicker infarct containing islands of viable cardiac tissue. CS represents non-diabetic Control Sham, DS represents Diabetic Sham, CLZMI represents non-diabetic Control animals that received Ad.LacZ injections, DLZMI represents Diabetic animals that received Ad.LacZ injections, CVAMI represents non-diabetic Control animals that received combination gene therapy, DVAMI represents Diabetic animals that received combination gene therapy.

Figure 3: Effect of combination gene therapy on capillary and arteriolar density 7 days after the intervention. (A) Representative images and (B) Graphical representation of capillary density analysis among the different groups (counts/mm²). (C) Representative images and (D) Graphical representation of arteriolar density analysis among the different groups (counts/mm²). There was a significant increase in the capillary and arteriolar density in the CVAMI and DVAMI groups when compared to the CLZMI and DLZMI groups, respectively. CS (black solid bars) represents non-diabetic Control Sham, DS (grey solid bars) represents Diabetic Sham, CLZMI (white bar) represents non-diabetic Control animals that received Ad.LacZ injections, DLZMI (diagonal brick bar) represents Diabetic animals that received Ad.LacZ injections, CVAMI (wave bar) represents non-diabetic Control animals that received combination gene therapy, DVAMI (wide upward diagonal bar) represents Diabetic animals that received combination gene therapy. Values given as mean ± SEM. *p ≤ 0.05 when DS is compared to CS, †p ≤ 0.05 when compared to CLZMI, ‡p ≤ 0.05 when DVAMI is compared to DLZMI.

Figure 4: Effect of combination gene therapy on the expression of VEGF, Flk-1, Ang-1, Tie-2 and survivin 4 days after the intervention. (A) Representative Western blots of VEGF, Flk-1, Ang-1, Tie-2 and survivin comparing CS and DS groups. (B) Bar graphs show the quantitative difference in expression of VEGF, Flk-1, Ang-1, Tie-2 and survivin respectively in between the CS and DS groups. (C) Representative Western blots for VEGF, Flk-1, Ang-1, Tie-2 and
survivin comparing the CLZMI, DLZMI, CVAMI and DVAMI groups, 4 days after the therapy. (D) Bar graphs show the quantitative difference in expression of VEGF, Flk-1, Ang-1, Tie-2 and survivin respectively in between the CLZMI, DLZMI, CVAMI and DVAMI groups. There was significant increase in the expression of these proteins in the CVAMI and DVAMI groups when compared to the CLZMI and DLZMI groups respectively. GAPDH was used as loading control. CS (black solid bars) represents non-diabetic Control Sham, DS (grey solid bars) represents Diabetic Sham, CLZMI (white bar) represents non-diabetic Control animals that received Ad.LacZ injections, DLZMI (diagonal brick bar) represents Diabetic animals that received Ad.LacZ injections, CVAMI (wave bar) represents non-diabetic Control animals that received combination gene therapy, DVAMI (wide upward diagonal bar) represents Diabetic animals that received combination gene therapy. Values given as mean ± SEM. *p ≤ 0.05 when DS is compared to CS, †p ≤ 0.05 when compared to CLZMI, †p ≤ 0.05 when DVAMI is compared to DLZMI.

**Figure 5:** Effect of combination gene therapy on phosphorylation of MK2 (Western blot) and DNA binding activity of NFκB (EMSA) (A) Representative Western blots for p-MK2 comparing CS and DS groups, 2 and 4 days after the surgery. (B) Graphical representation of p-MK2 in the CS and DS groups, 2 and 4 days after the therapy. (C) Representative Western blots for p-MK2 comparing CLZMI, DLZMI, CVAMI and DVAMI groups, 2 and 4 days after the therapy. (D) Graphical representation of p-MK2 in the CLZMI, DLZMI, CVAMI and DVAMI groups, 2 and 4 days after the therapy. The gene therapy significantly increased the levels of p-MK2, 2 and 4 days after the therapy. (E) EMSA analysis reveals increased DNA binding activity of NFκB in the CVAMI and DVAMI groups when compared to the CLZMI and DLZMI groups respectively. CS (black solid bars) represents non-diabetic Control Sham, DS (grey solid bars) represents Diabetic Sham, CLZMI (white bar) represents non-diabetic Control animals that received Ad.LacZ injections, DLZMI (diagonal brick bar) represents Diabetic animals that received Ad.LacZ injections, CVAMI (wave bar) represents non-diabetic Control animals that received combination gene therapy, DVAMI (wide upward diagonal bar) represents Diabetic animals that received combination gene therapy. Values given as mean ± SEM. *p ≤ 0.05 when DS is compared to CS, †p ≤ 0.05 when compared to CLZMI, †p ≤ 0.05 when DVAMI is compared to DLZMI.

**Figure 6:** Effect of combination gene therapy on left ventricular myocardial functions (echocardiography). (A) Representative echocardiograph pictures of parasternal short axis images after 30 days of surgery and therapy. Bar graphs represent: (B) Left Ventricular Inner Diameter in systole (LVIDs, in mm), (C) % Ejection Fraction-EF, (D) % Fractional Shortening-FS and (E) Heart rate in beats/minute. There was a significant increase in the myocardial functions in the CVAMI and DVAMI groups when compared to the CLZMI and DLZMI groups, respectively. CS (black solid bars) represents non-diabetic Control Sham, DS (grey solid bars) represents Diabetic Sham, CLZMI (white bar) represents non-diabetic Control animals that received Ad.LacZ injections, DLZMI (diagonal brick bar) represents Diabetic animals that received Ad.LacZ injections, CVAMI (wave bar) represents non-diabetic Control animals that received combination gene therapy, DVAMI (wide upward diagonal bar) represents Diabetic animals that received combination gene therapy. Values given as mean ± SEM. *p ≤ 0.05 when DS is compared to CS, †p ≤ 0.05 when compared to CLZMI, †p ≤ 0.05 when DVAMI is compared to DLZMI.
Figure 1

A  Control Sham  Ad-LacZ Sham

B  VEGF
   CS  CLZMI  CVAMI
   DS  DLZMI  DVAMI

C  Ang-1
   CS  CLZMI  CVAMI
   DS  DLZMI  DVAMI
Figure 2

7 Days
A
CS  CLZMI  CVAMI
DS  DLZMI  DVAMI

30 Days
B
CS  CLZMI  CVAMI
DS  DLZMI  DVAMI
Figure 3

A

B

C

D

Rescue of diabetic myocardium by VEGF/Ang-1 Therapy
Figure 4

(A) 4 Days

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(B) Arbitrary Units x 10^3

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(C) 4 Days

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(D) Arbitrary Units x 10^3

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Rescue of diabetic myocardium by VEGF/Ang-1 Therapy
Figure 5

A

2 Day  4 Day

CS/DS  CS/DS  CS/DS  CS/DS

p-MK2  MK2

B

p-MK2

CS  DS  CS  DS

2 Day  4 Day

Supershift

NFκB

C

2 Day  4 Day

CL-2M1  DL-2M1  CVAM1  DVAM1  CL-2M1  DL-2M1  CVAM1  DVAM1

p-MK2  MK2

D

2 Day  4 Day

CL-2M1  DL-2M1  CVAM1  DVAM1  CL-2M1  DL-2M1  CVAM1  DVAM1

p-MK2

Arbitrary Units x 10^5

E
Figure 6

A: Images showing myocardium with different conditions.

B: Graph showing LV end-diastolic diameter (mm) with conditions CS, DS, CLZMI, DLZMI, CVAMI, and DVAMI.

C: Bar chart for Ejection Fraction (%) with conditions CS, DS, CLZMI, DLZMI, CVAMI, and DVAMI.

D: Bar chart for Fractional Shortening (%) with conditions CS, DS, CLZMI, DLZMI, CVAMI, and DVAMI.

E: Graph for Heart Rate (Beats/min) with conditions CS, DS, CLZMI, DLZMI, CVAMI, and DVAMI.