Detection of Placenta Growth Factor (PlGF) Protein in Neonatal Rat Cardiomyocytes

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ABSTRACT

Angiogenesis, the formation of blood vessels, can provide blood to the heart when its normal arteries are compromised. Angiogenesis is highly dependent on the Vascular Endothelial Growth Factor (VEGF) family. Placental Growth Factor (PlGF) is a member of this family and is crucial for pathological angiogenesis in the adult. PlGF coupled with the more common VEGF could greatly increase angiogenesis in the heart tissue, thus providing oxygen to ischemic heart tissue. Previous research has shown that PlGF mRNA increases with six hours of hypoxia which models ischemia. However, not much is known about PlGF expression on the protein level. We intend to establish that PlGF protein expression will increase in rat cardiomyocytes which have undergone 6, 12 and 24 hours of hypoxia compared to normoxic rat cardiomyocytes. This information will later be used in studies of potential protective treatments using PlGF.

BACKGROUND

Myocardial ischemia/infarction is a leading cause of morbidity and mortality in the United States. Although therapies to enhance coronary angiogenesis could substantially reduce the severity of ischemic heart disease, the endogenous mechanisms promoting blood vessel growth in the heart are not well known. One hypothesis involves the production of specific factors from cardiomyocytes that then act in a paracrine fashion to induce the growth of the surrounding vasculature.

There is considerable evidence that members of the vascular endothelial growth factor (VEGF) family play an important role in the establishment, growth, and maintenance of blood vessels under a variety of physiological and pathological conditions (Ferrara et al., 2001). One VEGF family member that has received relatively little attention in terms of its expression and function in heart tissue is placenta growth factor (PlGF). PlGF has been shown to act synergistically with vascular endothelial growth factor (VEGF) to promote angiogenesis and inhibit apoptosis. Although we and others have shown significant upregulation of VEGF expression in models of coronary angiogenesis (Torry et al., 2001), quantitative changes in PlGF expression are not known.

The present study sought to determine: 1) if cardiomyocytes express PlGF protein, 2) if hypoxia alters PlGF protein expression in cardiomyocytes.

METHODS

Rat neonatal cardiomyocyte cell culture:

Rat neonatal cardiomyocytes were isolated and cultured as described (Zheng et al., 2001). Purity of cultures were assessed by indirect immunocytochemistry using monoclonal antibody against sarcomeric myosin (MF-20; Univ Iowa Hybridoma Center) and propidium iodine counterstain.

Culture Treatments:

Hypoxic conditions were established by placing cell cultures in an anaerobic pouch (BBL), and reoxygenated by removing the cultures from the anaeroabic pouch. Hypoxia times were 1, 3, 6, 12, 24 hours with each cell culture allowed to reoxygenate for ten minutes.

PlGF mRNA (RT-PCR) and protein (Western blot) expression:

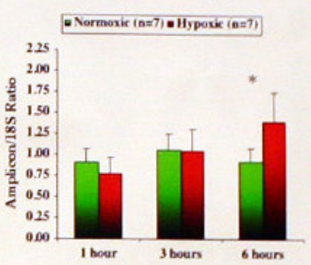
a. RT-PCR: Total RNA (500ng) was reverse transcribed (Amersham Pharmacia) and 2ul were subjected to cycles of PCR in the linear range of amplification. Relative differences in PlGF mRNA expression were assessed by semi-quantitative multiplex RT-PCR (Torry et al., J. Mol. Cell. Cardiol., 2001) using the following primers (50pmol): 18S classic (488bp) or classic II (324bp) products (Ambion, Inc.); PlGF (224 bp product). The resultant products were separated by electrophoresis on a 2.0% agarose, stained with ethidium bromide and visualized with Kodak Gel Documentation System (1D-EDAS). Densitometry was performed and the ratio of amplicon to 18S was computed and compared statistically.

b. Western blot:After resolution by 10% SDS-PAGE, PlGF proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (insert brand) at 100 V for 45 minutes. PVDF membranes were probed with PlGF (R-18) affinity-purified goat polyclonal antibody raised against the carboxy terminus of PlGF precursor of rat origin (1:1000; Santa Cruz Biotechnology, Inc.). Donkey anti-goat HRP-conjugated secondary antibody (1:200,000; Dakopatts) was used in conjunction with Chemiluminescent Substrate (Pierce) and film exposure to visualize the immunoreaction.

RESULTS

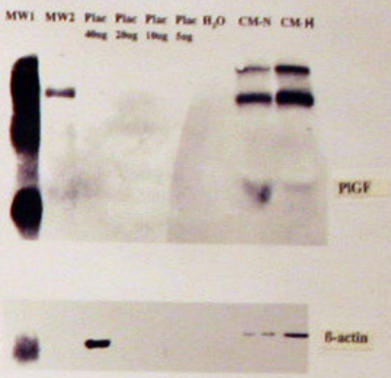
PlGF mRNA Expression in Cultured Neonatal Rat Cardiomyocytes

Cardiomyocytes were isolated from neonatal rats and cultured in DMEM/10% FBS. Cells were cultured under hypoxic (<2%O_2) or normoxic conditions for 1, 3, or 6 hours. Total RNA was isolated immediately and 500ng of total RNA was reverse transcribed. Resulting cDNA (2 uL) was subjected to multiplex PCR amplification using primers for rat VEGF, PlGF, VEGFR-1, and VEGFR-2, with 18S as an internal standard within the linear range of amplification for each primer. Amplicon/18S ratios were determined and fold changes in hypoxic compared to normoxic cultures within each time point were calculated. *p = 0.06 from Normoxia.

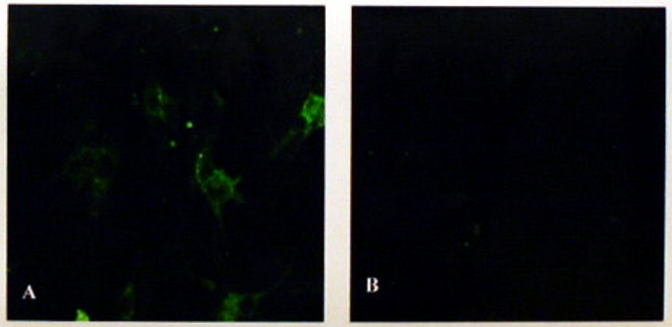


Western blot: PlGF Protein Expression in Hypoxic Cells

Cardiomyocytes were isolated from neonatal rats and cultured in DMEM/10% FBS. Cells were cultured under hypoxic (<2%O_2) or normoxic conditions for 1, 3, 6, 12 or 24 hours. Cells were lysed and stored at -80 degrees Celsius. In the above Western Blot, 55.7 ug of protein from normoxic cardiomyocyte lysates and 50.6 ug of protein from lysates of cardiomyocytes which were placed under 6 hours of hypoxia were loaded. The membrane was probed with GT anti-rat PlGF primary antibody and DK anti-GT antibody with HRP, then exposed to film and developed. Next, the membrane was stripped and probed with mouse anti-ß-actin primary antibody and rabbit anti-mouse secondary antibody. This served as an internal standard since all protein loaded should contain ß-actin.



Cultured Neonatal Rat Cardiomyocytes



Cardiac cells were isolated from 2-3 day old rats, selectively plated to enrich cardiomyocytes or non-cardiomyocytes. Representative aliquots were collected and cultured on chamber slides in DMEM/10% FBS for 48hrs. Panel A: The cultures were washed, fixed with -20°C acetone, re-hydrated in PBS and reacted with goat polyclonal antibody to rat PlGF (Santa Cruz Biotechnology) and then FITC-conjugated donkey anti-goat IgG antibody (Dakopatts). Panel B: PBS was substituted for the primary antibody and the cultures were reacted with the FITC-conjugated donkey anti-goat IgG antibody.

SUMMARY AND CONCLUSION

PlGF mRNA expression in isolated rat cardiomyocytes:

1. PlGF mRNA expression was present in both normoxic and hypoxic cells at all t time periods.

2. Hypoxia tended to increase PlGF mRNA expression but expression was significantly increased after 6 hours of hypoxia.

Immunocytochemistry:

PlGF protein is present in the cytoplasm of neonatal rat cardiomyocytes.

Western Blot:

We could not reliably detect cardiomyocyte PlGF protein expression by Western Blc is too early to claim results but recent analysis appears encouraging; we are contin to optimize the procedure.

Conclusion:

Western Blot detection of PlGF protein is still being sought. We are currently strivi find the correct antibody dilution to be able to develop the Western Blots. From t we will run Western Blots of hypoxic and normoxic cardiomyocytes in hopes to d differences in PlGF expression.

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