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Circulating Neuregulin-1β Levels Vary According to the Angiographic Severity of Coronary Artery Disease and Ischemia

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Abstract

Background—Coronary artery disease (CAD) is the leading killer in the U.S. Patients with severe CAD and ischemia have worse prognosis. Therefore expansion of biomarker research, to identify at risk individuals and explain the complex biology between cardiovascular growth factors and atherosclerosis is needed. Neuregulin-1β (NRG-1β) is a myocardial stress activated growth and survival factor released from endocardial and endothelial cells. NRG-1β is essential for cardiovascular development and a regulator of angiogenesis. We postulated that plasma and serum levels of NRG-1β would vary in relation to CAD severity and the presence of stress-induced ischemia.

Methods—We measured serum and plasma levels of NRG-1β and vascular endothelial growth factor (VEGF) in 60 patients undergoing cardiac catheterization. CAD severity was calculated from angiographic results using a modified Duke jeopardy score.

Results—Serum NRG-1β (sNRG-1β), plasma NRG-1β (pNRG-1β), serum VEGF (sVEGF), and plasma VEGF (pVEGF) were detectable in the majority of patients. The pNRG-1β levels were approximately two fold higher than sNRG-1β. Both sNRG-1β and pNRG-1β correlated inversely with CAD severity. Plasma NRG-1β levels were statistically higher in patients with stress-induced ischemia denoted by a positive myocardial perfusion imaging study that correlated with angiographic findings ($p = 0.02$).

Conclusions—Both serum and plasma NRG-1β correlated inversely with angiographic severity of CAD. Plasma NRG-1β levels were two fold higher than serum and were higher in patients with stress-induced ischemia. Therefore we conclude that plasma is the optimal source for the further exploration of the biological significance of NRG-1β as a biomarker of CAD severity and ischemia.

Keywords

biomarkers; growth factors; atherosclerosis; stress-induced ischemia; heregulin

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Introduction

The severity of coronary artery disease (CAD) relates to several biological factors such as inflammatory cytokines and lipoproteins that subsequently influence atherosclerosis and angiogenesis. The relationship between vascular growth factors and CAD severity is poorly understood. A validated biomarker that reflects CAD severity and ischemic burden would be an important screening tool to facilitate the management of patients with cardiac risk factors and cardiac symptoms.

Neuregulin (NRG-1β) is a novel stress activated cardiac growth and angiogenic factor, which is activated by ischemia and exercise in animals [1, 2]. NRG-1β acts through ErbB receptors to regulate cell survival, growth, metabolism, as well as angiogenesis [1, 3–5]. We developed an assay to quantify circulating NRG-1β in the serum (sNRG-1β) [6]. Higher sNRG-1β levels were associated with worse symptomatic heart failure (HF) and poor prognosis, particularly in ischemic heart disease [7]. These results suggest that further work is needed to understand NRG-1β's relationship with ischemia.

Vascular endothelial growth factor (VEGF), a potent initiator of angiogenesis, is rapidly up-regulated in animal models of myocardial ischemia and induced by neuregulin [8, 9]. Systemic and intracardiac VEGF levels in samples from patients with CAD have been measured previously with variable results [10–14].

The primary objective of this study was to determine if plasma/serum NRG-1β levels were associated with CAD severity or differed in the presence of ischemia in a cross-sectional sampling of patients who underwent coronary angiogram. The secondary objective was to further characterize the relationship of NRG-1β and VEGF as paracrine regulator of angiogenesis [15]. We postulated in our null hypothesis that there is no variation in NRG-1β levels by CAD severity and no difference due to ischemia. A rejected null hypothesis suggests that NRG-1β has a biological association with atherosclerosis and might serve as a potential vascular biomarker reflecting CAD severity and ischemia in patients with cardiovascular risk factors.

Methods

Study Patients

The study population consisted of a retrospective cohort of 60 patients who underwent coronary angiography due to either chest pain and/or a positive stress test from September 2004 – May 2005 from a single urban, academic referral center. Patients were selected and divided into 3 groups according to the severity of CAD: 20 consecutive patients with angiographically normal coronary arteries, 20 with >50% stenotic disease in at least one major coronary artery branch and 20 with occluded disease in at least one major coronary artery branch. The CAD severity was further characterized in these subgroups using the modified Duke jeopardy score, which accounts for lesion location, degree of stenosis, and number of vessels involved. Severity score of 0 = no angiographic CAD, 2 = mild CAD, 4–6 = moderate CAD, and 8–12 = severe CAD [16]. Stress-induced ischemia was identified in those patients who underwent a nuclear myocardial stress test with perfusion mismatch or diagnostic EKG changes that correlated with angiographic findings of a hemodynamically significant lesion in the corresponding location. Collateral arteries were noted during angiography when territories were supplied by alternative artery distal to a significant stenosis in a major coronary vessel.

Exclusion criteria were atrial fibrillation, impaired left ventricular function (ejection fraction <45%), significant valvular heart disease, recent (<3 months) ischemic stroke, renal failure, hepatic impairment, chronic obstructive pulmonary disease, neoplasm, connective tissue disease, infections, or steroid use prior to cardiac catheterization. The Institutional Review Board approved the study and all participants signed an informed consent allowing blood samples to be stored and used for future cardiac studies.

Collection of Samples

Arterial blood samples were collected in vacuum tubes containing no additives and in vacuum tubes containing EDTA from a 6F arterial sheath prior to any anticoagulation. Samples were drawn from a femoral arterial sheath to avoid unnecessary peripheral blood draws and to standardize processing of blood samples. After centrifugation at 3,000 rpm for 10 min at room temperature, serum and plasma were separated, stored in aliquots and kept frozen at −80° C until measurement. Plasma samples were centrifuged within 30 minutes of collection, and serum samples were centrifuged after 30 minutes at room temperature. Frozen serum and plasma samples were thawed for VEGF and NRG-1β in a single run.

Description of ELISA's: VEGF and NRG-1β

Growth factors were measured by enzyme-linked immunoassays (ELISA) as follows: A commercially available Quantikine ELISA kit from R&D Systems, Minneapolis, MN (cat#DVE00) was used to quantify VEGF levels. Both plasma and serum samples were diluted (1:4 in PBS).

We measured NRG-1β levels using DuoSet ELISA development system (R&D cat# DY377) [7]. Briefly a 96 well plate (Pierce cat # 15041) was coated with the capture antibody overnight at room temperature on a plate shaker. Capture antibody was washed and the plate was blocked with blocking buffer as described in the package insert. A standard curve was generated using the lyophilized NRG-1β according to manufacturer's instructions with 2% normal goat serum (Invitrogen: cat # PCN5000). The standards and diluted samples (1:3 in PBS) were added to each well and incubated for 2 hours. After serial washing, the detection antibody was added, incubated at room temperature and then washed. Streptavidin-HRP was added and incubated on the plate shaker for 30 minutes at room temperature. The plate was washed and then the substrate solution (Thermo Scientific 1-Step Ultra TMB-ELISA cat # 34028) was added for 10 minutes protected from light. Sulfuric acid stop solution was added and the absorbance was read at 450nm using a spectraphotometric plate reader.

Aliquoted samples were run in duplicates and the average of the two values were used in the analysis. The detection limit for VEGF ranged from 31.2 – 2,000 pg/ml. The average intra-assay coefficient of variation for serum VEGF was 9.7% and plasma VEGF was 13.5 % with inter-assay coefficient of variation for all VEGF < 14.0%. Serum and plasma NRG-1β detection limit ranged from 0.3 to 30 ng/ml. Samples below the detectable limit (n = 12 serum and n = 0 plasma) were assigned a value half-way between 0 and lowest limit (0.15 ng/ml). The average intra-assay coefficient of variation for serum NRG-1β was 5.6% and plasma NRG-1β 3.9% with inter-assay coefficient of variation for all NRG1β < 7.0%.

Statistical analysis

Continuous variables were summarized as mean± SD. Kruskal-Wallis test was used to test for a difference between multiple CAD severity groups and Mann-Whitney U test was used to detect a difference between two groups. Categorical variables were summarized as count and percentage and compared by Chi-square test between multiple CAD severity groups. VEGF and NRG-1β values were non-normally distributed based on Shapiro-Wilks test and therefore the median values and ranges were also reported in addition to the standard

deviation (± SD). Spearman's correlation coefficient was calculated and tested between serum and plasma VEGF and NRG-1β and continuous cardiac risk factors. A p value < 0.05 was considered statistically significant. Due to the discovery nature of the study, multiple comparisons are not adjusted and further confirmatory studies are needed. Statistical analysis was performed with statistical software R (www.r-project.org) and SPSS Statistics version 17.0 (SPSS Inc, Chicago Illinois).

Results

Characteristics of study patients

Baseline characteristics showed that aspirin was used more frequently in groups with CAD (mild Duke score = 2, moderate Duke score = 4–6, and severe Duke score 8 –12) (p = 0.01) than patients without CAD (none Duke score = 0). No other clinically significant differences were seen between groups with varying CAD severity (Table 1). Although not statistically different within the Duke severity score groups, more than half of all patients were on a statin medication. The high percentage of statin use reflects a higher risk population that was referred for cardiac catheterization.

Serum and Plasma VEGF/NRG-1β and Severity of CAD

There was a statistically significant correlation between serum and plasma NRG-1β demonstrating an association with lower circulating NRG-1β in the presence of more diffuse coronary artery disease (sNRG-1β rho = 0.363, p = 0.004 and pNRG-1β rho = 0.261, p = 0.044) (Figure 1). There was no statistically significant association with serum or plasma VEGF and CAD severity. Serum and plasma NRG-1β and VEGF were non-normally distributed with a trend toward lower levels in the presence of more severe CAD (Table 2).

Difference between Serum and Plasma VEGF/NRG-1β and Stress-induced Ischemia

Levels of pNRG-1β were higher in the group with ischemia (n = 23) (median 4.1 ng/ml ± 2.2 ng/ml) than in the group without ischemia (pNRG-1β in absence of stress-induced ischemic group n = 37 median 3.3 ng/ml ± 1.9 ng/ml, p = 0.02). No statistical differences were observed in sNRG-1β, sVEGF, or pVEGF in the presence or absence of stress-induced ischemia, however there was a trend toward higher mean sNRG-1β in patients with stress-induced ischemia (Table 3).

Correlations between VEGF, NRG-1β and Collateral Coronary Arteries

No significant correlation was observed between sVEGF and sNRG-1β (rho = 0.121, p = 0.356), or pVEGF and pNRG-1β (rho = 0.058, p = 0.657). No correlation was observed between collateral artery presence and sNRG-1β, pNRG-1β, sVEGF, or pVEGF.

Discussion

The principle finding of this study was that in patients with stable CAD serum and plasma NRG-1β inversely correlated with CAD severity. Plasma NRG-1β levels were detectable in all participants and were two-fold higher than serum, making plasma the optimal source for detecting circulating NRG-1β. Additionally plasma NRG-1β levels were statistically higher in patients with stress-induced ischemia, supporting the notion that circulating NRG-1β is induced by cardiac stress and perfusion mismatch related to atherosclerosis. Similarly we observed in a heart failure cohort of nearly 900 patients that patients with ischemic heart failure (HF) had higher circulating NRG-1β levels than patients with other causes HF [7]. Additionally patients with ischemic HF had increased risk of death or transplantation suggesting that NRG-1β is a potentially useful biomarker corresponding with ongoing cardiovascular ischemic stress that has prognostic significance. The study confirms that in a

mixed cohort of patients ranging from relatively healthy patients with few cardiac risk factors to severe CAD, circulating NRG-1β is detectable in measurable amounts. While larger studies are needed to understand the biological significance of circulating NRG-1β in the progression of atherosclerosis, and acute coronary syndromes, there are plausible explanations for higher levels of pNRG-1β due to ischemia and lower levels in more severe CAD.

The association between reduced circulating NRG-1β in the presence of more severe CAD may be an indication of endothelial dysfunction. NRG-1β is released from vascular endothelial cells and exerts paracrine effects on local myocytes and blood vessels [5]. It is well known that preceding angiographically evident CAD, endothelial dysfunction can be demonstrated with altered vasoreactivity and decreased nitric oxide production. Thus, one explanation for our findings is that as CAD progresses endothelial cells have impaired NRG-1β release as a manifestation of this dysfunction. In addition, severe CAD is known to be a state of increased inflammation. Inflammatory cytokines decrease total NRG-1β transcript and expression level, in vitro (unpublished data, Cote and Sawyer), providing another explanation for the relationship between NRG-1β levels and CAD severity. Previous works has established that NRG-1β is expressed in atherosclerotic lesions in human coronaries and carotid arteries [17–19]. Recent studies demonstrated that NRG-1β was atheroprotective by suppressing macrophage foam cell formation in plaques [20]. Additionally they showed that in ApoE knock out mice that a chronic infusion of NRG-1β decreased atherosclerosis in the aorta while anti-NRG-1β antibody (aka Her2+ receptor antibody) accelerated aortic atherosclerosis. The current study supports the concept that endogenous NRG-1β plays a role in modulating severity of atherosclerosis.

There are other explanations for the lower NRG-1β level in patients with more advanced CAD. We have previously found that multiple NRG-1β isoforms are expressed in adult human and rat skeletal muscle. Exercise of skeletal muscle in rodents induces activation of NRG-1β/ErbB signaling [2]. In a cohort of healthy subjects, serum NRG-1β levels are positively correlated with cardio-respiratory fitness represented by maximum oxygen consumption (VO_2 max) [6]. Thus, lower NRG-1β levels in patients with significant CAD, especially those with occluded coronary arteries, may be attributable to reduced physical activity and release of skeletal muscle derived NRG-1β. In addition, sarcopenia is associated with advanced cardiac disease including CAD, and this may contribute to the current findings [21]. These observations expand our understanding of the relationship between NRG-1β and cardiovascular disease and set the foundation for future studies.

Our investigation also demonstrated that plasma concentrations of NRG-1β were statistically higher in patients with stress-induced ischemia. Previous work has shown oxidatitve stress, as well as ischemia/reperfusion promotes the proteolytic cleavage of NRG-1β from endothelial cells, which in turn promotes cell survival through paracrine signaling. NRG-1β expression can be up-regulated by cerebral ischemia and provides neuroprotective benefit decreasing the size of cerebral infarction in animals with ischemic stroke [19, 22]. Since NRG-1β is known to induce angiogenic factors, the presence of higher plasma levels in patients with stress-induced ischemia may reflect a cardioproetective mechanism signaling neovascularization and cardioprotection similar to its neuroprotective property. This study is the first to suggest that plasma NRG-1β is a detectable biomarker of stress-induced ischemia in humans and further studies are needed to explore the biological implications and clinical outcomes of these findings.

The biology of NRG-1β is complex and will require larger studies to clarify the effects of cardiac medications (i.e. aspirin, clopidogrel, beta-blockers, angiotensin converting enzyme nhibitor, angiotensin receptor blockers) on the measurement of NRG-1β as a biomarker. In

the current study there was no statistically significant difference in the cohort with regards to cardiac medications and severity of CAD. We hypothesize that cardiac medications that improve endothelial function and cardiovascular remodeling may result in increased endothelial cell NRG-1β expression reflecting an improvement in vascular health.

Although in vivo studies suggest that NRG-1β can regulate VEGF expression [15], we did not observe a correlation between sNRG-1β/sVEGF or pNRG-1β/sVEGF. In addition neither serum nor plasma VEGF varied by CAD severity. We did observe a statistically significant inverse correlation between serum and plasma NRG-1β and CAD severity. The correlation coefficient may have been improved with a more rigorous angiographic scoring system such as the SYNTAX or other scoring systems [23, 24]. The Duke jeopardy scoring system was selected for this study due fact it is easily calculated and accounts for major coronary factors that modify clinical outcomes (i.e. number of coronary vessels, location of stenosis, and severity of occlusion). The correlation coefficients may have been strengthened if the Duke jeopardy scoring system was not categorized, but classification system (mild, moderate, severe CAD) improved clinical utility.

In conclusion our results suggest that NRG-1β levels inversely correlates with CAD severity in stable patients and plasma NRG-1β levels are statistically higher in patients with stress-induced ischemia. Recently several biomarkers such as cystatin C and angiogenin demonstrate an association with severity of CAD similar to NRG-1β [25, 26]. NRG-1β also appears to be altered by the stress-induced ischemia, and therefore may have additional utility over other biomakers. The small sample size and baseline confounders prevent us from concluding a cause-effect association. Further studies are needed with larger sample sizes, prospective design, and patients with unstable CAD/acute infarctions to investigate if plasma NRG-1β is a reliable biomarker reflecting severity of CAD and burden of cardiac ischemia. NRG-1β biology is complex and will require more studies to understand its role as a surrogate biomarker of atherosclerosis and ischemia stress response but this study demonstrates that plasma is optimal source for future biomarker development.

Limitations

The convenient sample of 60 subjects with different CAD severity provides a relatively small sample size. The exclusion criteria limits the generalizability of our findings to other cohorts. The study was performed at a single center, and therefore subject to aggregation bias.

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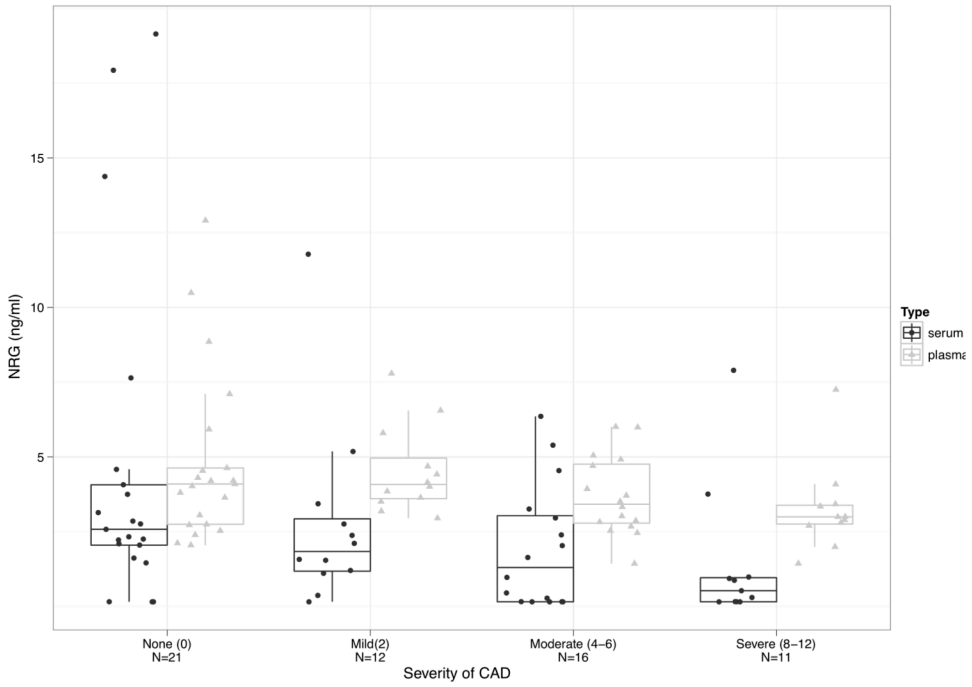

Figure 1.

Boxplots with mean and standard deviation of sixty patients who underwent coronary angiogram to assess CAD severity. CAD severity was classified into four groups using the modified Duke jeopardy score (none, mild, moderate, and severe CAD). Both serum and plasma NRG-1β levels were inversely associated with CAD severity (sNRG-1β rho = 0.363, p = 0.004, and pNRG-1β rho = 0.261, p = 0.044, Spearman's correlation).

Table 1

Baseline Cardiac Risk Factors and Medication Profile

	No CAD		Mild CAD		Moderate CAD		Severe CAD		
mean ± SD	**Duke Score 0**		**Duke Score 2**		**Duke Score 4 – 6**		**Duke Score 8 –12**		
number (%)	**n = 21**		**n = 12**		**n = 16**		**n = 11**		**p value**
Age	55	± 11	58	± 8	62	± 10	63	± 9	0.15
Body Mass Index	36	± 13	33	± 6	33	± 10	41	± 18	0.35
EF (%)	63	± 8	55	± 7	62	± 3	56	± 14	0.06
Males (%)	9	42.9%	10	83.3%	11	68.8%	7	63.6%	0.12
Hypertension	19	90.5%	10	83.3%	13	81.3%	8	72.7%	0.63
Hypercholesterolemia	15	71.4%	9	75.0%	12	75.0%	11	100.0%	0.28
Diabetes	6	28.6%	5	33.3%	4	25.0%	5	41.7%	0.71
Current Smoker	7	33.3%	7	58.3%	7	43.8%	7	63.6%	0.32
Family History CAD	13	61.9%	7	58.3%	7	43.8%	6	54.5%	0.73
Prior angioplasty/stent	0	0.0%	2	16.7%	3	18.8%	0	0.0%	0.09
Aspirin	10	47.6%	12	100.0%	13	81.3%	8	72.7%	0.01
Clopidogrel	3	14.3%	4	33.3%	6	37.5%	3	27.3%	0.41
Beta-Blocker	9	42.9%	7	58.3%	9	56.3%	5	45.5%	0.78
ACE	8	38.1%	4	33.3%	6	37.5%	5	45.5%	0.95
ARB	5	23.8%	3	14.3%	1	6.3%	0	0.0%	0.16
CCB	6	28.6%	3	25.0%	4	25.0%	2	18.2%	0.94
Diuretics	9	42.9%	6	50.0%	4	25.0%	5	45.5%	0.53
Statin	11	52.4%	9	75.0%	10	62.5%	9	81.8%	0.33

SD = standard deviation

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Table 2

Variation in VEGF and NRG-1β Levels by CAD Severity

median (range) ± SD	No CAD Duke Score 0 n = 21		Mild CAD Duke Score 2 n = 12		Moderate CAD Duke Score 4 – 6 n = 16		Severe CAD Duke Score 8 –12 n = 11	
serum VEGF (pg/ml)	176 (16 – 1538)	± 491	260 (16 –813)	± 294	200 (16 –822)	± 263	122 (16 –496)	± 209
plasma VEGF (pg/ml)	101 (16 –1399)	± 293	80 (30 –257)	± 728	237 (30 –744)	± 215	256 (55 –370)	± 120
serum NRG (ng/ml)	2.6 (0.2 –19.1)	± 5.5	1.8 (0.2 –11.8)	± 3.1	1.3 (0.2 –6.4)	± 2.1	0.5 (0.2 –7.9)	± 2.4
plasma NRG (ng/ml)	4.1 (2.0 –12.9)	± 2.9	4.0 (2.9 – 7.8)	± 1.5	3.4 (1.4 –6.0)	± 1.3	3.0 (1.4 –7.2)	± 1.5

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Table 3

Difference in VEGF and NRG-1β by Stress-Induced Ischemia

Median (range) ± SD	Positive Ischemia n = 23	Negative Ischemia n = 37	p value
serum VEGF (pg/ml)	247 (16 –1148) ± 312	163 (16 –1538) ± 384	0.40*
plasma VEGF (pg/ml)	131 (16 –1399) ± 298	103 (20 –744) ± 150	0.84*
serum NRG (ng/ml)	2.4 (0.2 –19.1) ± 4.3	1.6 (0.2 –17.9) ± 3.8	0.13*
plasma NRG (ng/ml)	4.1 (1.9 –12.9) ± 2.2	3.3 (1.4 –10.5) ± 1.9	0.02*

*Mann-Whitney U test