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Research Paper

Elevated Markers of Death Receptor-Activated Apoptosis are Associated with Increased Risk for Development of Diabetes and Cardiovascular Disease

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ABSTRACT

Background: An increased rate of cell death by apoptosis has been implicated in both diabetes and atherosclerosis. Apoptosis can be induced through activation of the death receptors TNF receptor 1 (TNFR-1), TRAIL receptor 2 (TRAILR-2) and Fas. Soluble forms of these receptors are found in plasma. The objective of this study was to determine if soluble death receptors are markers of receptor-activated apoptosis and predict risk for development of diabetes and cardiovascular events.

Methods: Fas ligand was used to induce apoptosis in peripheral blood mononuclear cells and INS-1 pancreatic β-cells and release of TNFR-1, TRAILR-2 and Fas measured by ELISA. Proximity Extension Assay was used to analyze plasma levels of TNFR-1, TRAILR-2 and Fas in baseline samples of 4742 subjects in the Malmö Diet and Cancer Study and related to development of diabetes and cardiovascular events during 19.2 years of follow-up.

Results: Activation of apoptosis by Fas ligand was associated with release of soluble Fas, TNFR-1 and TRAILR-2 in both cell types. Circulating levels of all three receptors were higher in subjects with diabetes and correlated with markers of impaired glucose metabolism in non-diabetic subjects. Among the latter, those in the highest tertile of soluble Fas, TNFR-1 and TRAILR-2 had increased risk for development of diabetes and cardiovascular events. These associations became weaker when adjusting for cardiovascular risk factors in Cox regression models, but remained significant for TRAILR-2 with incident diabetes, cardiovascular mortality, myocardial infarction and ischemic stroke, and for TNFR-1 with myocardial infarction.

Conclusion: The present study demonstrates an association between several cardiovascular risk factors and elevated levels of circulating markers of apoptotic cell death. It also shows that subjects with high levels of these biomarkers have increased risk of diabetes and CVD. This implies that soluble death receptors are markers of β-cell and vascular injury and potentially could be used as surrogate markers of therapeutic efficiency in risk factor interventions.

1. Introduction

Apoptosis is a genetically determined cell death program required to maintain cell homeostasis in the body but it has also been implicated in a number of different diseases including diabetes and cardiovascular disease (Hetts, 1998; Van Vre et al., 2012; Butler et al., 2003; Allen et al., 2005; Anuradha et al., 2014; Donath and Halban, 2004; Kolodgie et al., 1999; Crisby et al., 1997). Apoptosis can be triggered by intrinsic pathways induced by cellular stress and DNA damage or by extrinsic signals activating cell-surface death receptors. The death receptors are part of the tumor necrosis factor (TNF) receptor gene super family and include TNF receptor 1 (TNFR-1), TNF-related apoptosis-inducing ligand receptor 2 (TRAILR-2) and Fas (Ashkenazi and Dixit, 1998). These receptors have a common cytoplasmic sequence termed the "death domain" that transmits activation of caspase-8, which in turn induces apoptosis through activation of the effector caspases 3, 6 and 7. Several metabolic factors associated with diabetes have been shown to induce β-cell apoptosis including hyperglycemia and saturated fatty acids, whereas HDL has been attributed a protective effect (Federici et al., 2001; Maedler et al., 2001; Shimabukuro et al., 1998; Roehrich et al., 2003). One mechanism through which these factors enhance β cell apoptosis is through upregulation of Fas (Anuradha et al., 2014; Maedler et al., 2001). Metabolic stress is considered to be of key importance also in the vascular

Abbreviations: (TNFR-1), TNF receptor 1; (TRAILR-2), TNF-related apoptosis-inducing ligand receptor 2; (MDC), Malmö Diet and Cancer study; (PBMC), peripheral blood mononuclear cells; (MI), myocardial infarction.

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injury that drives development of atherosclerosis (Ross, 1999; Hansson, 2005). However, to what extent the higher incidence of cardiovascular disease (CVD) in diabetes is related to increased death receptor-activated apoptosis in the cardiovascular system in response to metabolic stress has not been extensively studied. The lack of circulating biomarkers has made it difficult to assess the clinical importance of death receptor-activated apoptosis for cardiovascular risk. We demonstrate here that induction of apoptosis through activation of Fas is associated with release of soluble TNFR-1, TRAILR-2 and Fas from cells suggesting that the circulating levels of these receptors can be used as biomarkers of apoptosis induced by activation of cell death receptors. Based on this observation we analyzed baseline plasma levels of soluble TNFR-1, TRAILR-2 and Fas in 4742 subjects participating the cardiovascular sub-cohort of the Malmö Diet and Cancer (MDC) study to investigate (Hetts, 1998) if metabolic changes characteristic for an impaired glucose metabolism are associated with elevated plasma levels of soluble TNFR-1, TRAILR-2 Fas and (Van Vre et al., 2012) if these markers of receptor-activated apoptosis predict risk for development of diabetes and cardiovascular events.

2. Methods

2.1. Receptor-Activated Release of Soluble Death Receptor and Apoptosis in Cultured Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were isolated from leukocyte concentrate from healthy donors using Ficoll Paque Plus (GE Healthcare). Cells were seeded at a density of 0.5×10^6 cells/well in complete RPMI (10 U/ml Penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate, 1% Hepes and 0,1% mercaptoethanol) with 2% human serum (Sigma Aldrich). The cells were exposed to IL-1β (10 ng/ml), soluble Fas ligand (0.5–5.0 μg/ml) and TNF-α (10–100 ng/ml) for 24 h in 37 °C with 5% CO_2 and the cell medium was subsequently analyzed by a multiplex assay detecting TNFR-1, TRAIL-R2 and Fas (R&D Systems, Minneapolis, MN, USA) and Luminex (Bio-Rad). The intra-assay and inter-assay CV for Luminex assays are <20% and <25%, respectively according to the manufacturer. The analytical range for the TNFR-1, TRAIL-R2 and Fas Luminex assays are 3–4803 pg/ml, 14–35,453 pg/ml and 25–53,758 pg/ml, respectively. For analyses of apoptosis, the cells were stained with Annexin V-PE and the viability marker 7-amino-actinomycin (7-AAD), and analyzed by flow cytometry according to the manufacturer's protocol (Biolegend). Early apoptotic cells were defined as AnnexinV^+/7-AAD^- and late apoptotic cells as AnnexinV^+/7-AAD^+.

INS-1837/13 cells (kindly provided by Dr. Pontus Dunér, Lund University), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, 50 mg/L streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM beta-mercaptoethanol. They were then stimulated with IL-1β, soluble Fas ligand and TNF-α and the release of TNFR-1, TRAIL-R2 and Fas analyzed as described above.

2.2. Study Population

The Malmö Diet and Cancer (MDC) study is a prospective population-based cohort (n = 28,449) study examining the association between diet and cancer (Berglund et al., 1993). Subjects born between 1926 and 1945 and living in Malmö were eligible for inclusion in the study. Between October 1991 and February 1994, every other participant was also invited to take part in a sub-study focusing on the epidemiology of carotid artery disease (MDC study cardiovascular cohort, n = 6103) (Hedblad et al., 2000). Out of these, 5405 came to a second baseline examination where fasting plasma samples were collected. We excluded 545 of these subjects from the present study due to incomplete clinical data and 118 subjects were further excluded because the analysis of their plasma samples did not pass the internal quality control for the biomarker analyses. The remaining 4742 subjects were followed from baseline examination until first event of cardiovascular disease (CVD), emigration from Sweden or death, up until December 31 st, 2013. Ascertainment of cases and validity of the registries used (the Swedish Discharge Registry, the Stroke Register of Malmö and the Cause of Death Registry of Sweden) have been proven to be high. A coronary event was defined as a fatal or non-fatal myocardial infarction on the basis of the International Classification of Diseases 9th and 10th revisions (ICD-9 and ICD-10) codes 410 and I21, respectively. Death due to ischemic heart disease was defined based on codes 412 and 414 (ICD-9) or I22, I23 and I25 (ICD-10). Cases with a first ischemic stroke were identified from the Malmö stroke registry, which has continuously monitored and searched for stroke cases in both inpatient and outpatient care since 1989 (Zia et al., 2007). Stroke diagnoses registered before December 31 st, 2010 were validated by review of the medical records. Strokes registered after this date were not included in the analysis due to lack of validation. Ischemic stroke was defined as ICD-9 codes 434 or 436 or ICD-10 codes I63 or I64 and hemorrhagic stroke as ICD-9 codes 430 or 431 or ICD-10 codes I60 or I61. Incident diabetes was identified by the Malmö HbA1c register, the Swedish National Diabetes Register, the Swedish inpatient register, the Swedish outpatient register, the nationwide Swedish drug prescription register, and the regional Diabetes 2000 register of the Skåne region as previously described (Muhammad et al., 2016). Diabetes at baseline was defined as a history of diabetes or an over-night fasting whole blood glucose of ≥6.1 mmol/l according to the WHO criteria (Alberti and Zimmet, 1998). Hypertension was defined as blood pressure ≥ 140/90 mm Hg or blood pressure lowering medication, hypercholesterolemia as >5 mmol/l, smoking as current smoking. Blood pressure, smoking habits and, hsCRP and circulating lipoprotein lipid levels were determined as previously described (Hedblad et al., 2000). The study was approved by the Regional Ethical Review Board in Lund (LU 51–90) and was conducted in accordance with the Helsinki Declaration. All subjects gave written consent. The reporting of this cohort study is in accordance with the STROBE guidelines.

2.3. Analysis of Soluble Death Receptor in Plasma

Plasma levels of TNFR-1, TRAIL-R2 and Fas were analyzed by the Proximity Extension Assay technique using the Proseek Multiplex CVD^{96x96} reagents kit (Olink Bioscience, Uppsala, Sweden) at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala. Oligonucleotide-labeled antibody probe pairs were allowed to bind to their respective targets present in the plasma sample and addition of a DNA polymerase led to an extension and joining of the two oligonucleotides and formation of a PCR template. Universal primers were used to pre-amplify the DNA templates in parallel. Finally, the individual DNA sequences were detected and quantified using specific primers by microfluidic real-time quantitative PCR chip (96.96, Dynamic Array IFC, Fluidigm Biomark). The chip was run with a Biomark HD instrument. The CV for intra-assay variation (within-run) and inter-assay variation (between-run) for TNFR-1, TRAIL-R2 and Fas are 8% and 12%, 10% and 5%, 8% and 12%, respectively and the analytical ranges 7.1–31,250 ng/ml, 0.2–7812 ng/ml and 1.0–15,625, respectively. Data analysis was performed by a preprocessing normalization procedure using Olink Wizard for GenEx (Multid Analyses, Sweden). All data are presented as arbitrary units (AU). General calibrator curves to calculate the approximate concentrations as well as technical information about the assays are available on the Olink homepage (http://www.olink.com).

2.4. Genotyping, Quality Control, Genome Wide Association Analyses (GWAS)

Genotyping was performed using *Illumina* HumanOmniExpress BeadChip v. 1 at Broad Institute, Cambridge, MA USA. During the quality control procedures (QC) we removed individuals having a call rate of

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