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Diabetes modifies the relationships among carotid plaque calcification, composition and inflammation

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ABSTRACT

Background and aims: Diabetes is traditionally associated with vascular calcification, but the molecular mechanisms are largely unknown. We herein explored the relationships among carotid plaque calcification, composition and gene expression, and how these are modified by diabetes.
Methods: We collected carotid endoarterectomy specimen from 59 patients, of whom 23 had diabetes. We analysed histology with pentachromic staining, calcification with Alizarin red and Von Kossa's staining, chemical calcium extraction and quantification, as well as gene expression by quantitative PCR.
Results: We detected no differences in the extent of plaque calcification and in plaque composition between diabetic and non-diabetic patients. In non-diabetic plaques, calcium content was directly correlated with the area occupied by muscle/fibrinoid tissue and inversely correlated with collagen, but such correlations were not seen in plaques from diabetic patients. While consistent correlations were found between calcium content and RUNX2 (direct), as well as Osteopontin (inverse), diabetes modified the association between plaque calcification and inflammatory gene expression. Only in diabetic plaques, calcium content was inversely correlated with MCP1 and IL1b, whereas the direct correlation with TNF-alpha expression seen in non-diabetic plaques was lost in diabetes.
Conclusions: Though plaque composition and calcification were not quantitatively affected, diabetes modified the relationships between plaque calcium, composition and inflammation. These results suggest that the mechanisms and the clinical significance of atherosclerotic calcification in diabetic may be different than in non-diabetic patients.

1. Introduction

Diabetes accelerates atherosclerosis, through the negative effects exerted by hyperlgycemia and the associated biochemical abnormalities on vascular wall cells and inflammatory processes [1–3]. Cardiovascular (CV) diseases is the major causes of death in diabetic patients [4] and understanding what drives excess CV morbidity and mortality in diabetes is a major health care challenge. One typical feature of diabetic vascular disease is ectopic calcification, which can occur in the medial layer of large and medium-size arteries, as well as in the intima of atherosclerotic lesions [5]. Unfortunately, the mechanisms driving excess calcification in diabetes are largely unknown, thereby limiting the identification of therapeutic or preventive strategies. While medial calcification can increase CV morbidity by inducing stiffness and raising cardiac post-load, the clinical meaning of atherosclerotic calcification is less clear [6]. Largely calcified plaques may be more stable, whereas microcalcifications typically identify unstable or culprit lesions [7]. In addition, there is a paucity of studies comparing the clinical and histopathological correlates of atherosclerotic calcification in diabetic versus non-diabetic patients. Inflammation is believed to play a major role in ectopic vascular calcification [8,9], but hyperglycemia, the specific biochemical feature present only in diabetes, also directly affects osteogenesis programmes [10–12]. We therefore speculate that the mechanisms driving vascular calcification in diabetic and

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in non-diabetic patients may be different. In this study, we analysed carotid endarterectomy (CEA) specimens from diabetic and non-diabetic subjects patients to evaluate the relationships among plaque calcium content, tissue composition and gene expression.

2. Materials and methods

2.1. Patients

The protocol was approved by the local Institutional Review Board. All participants were recruited at the time of carotid endoarterectomy (CEA) at the Division of Vascular Surgery of the University Hospital of Padova. We collected the following data from electronic hospital charts: age, sex, prevalence of obesity (defined as a body mass index $\geq$30 kg/m^2), hypertension (defined as a systolic blood pressure >140 mm Hg or a diastolic blood pressure >90 mm Hg, or the use of anti-hypertensive medications), dyslipidemia (defined as pre-statin total cholesterol level >240 mg/dL, or a LDL cholesterol level >130 mg/dL, or a triglycerides level >200 mg/dL), current or past smoking habit (of 1 or more cigarettes per day). Diabetes was defined based on fasting plasma glucose ($\geq$126 mg/dl), or HbA1c ($\geq$6.5%), or the use of anti-diabetic medications. Prevalent coronary artery disease was defined as a past history of myocardial infarction or angina in the presence of >50% epicardial coronary artery stenosis. Cerebrovascular disease was defined as a past history of stroke or transient ischemic attack. Data on medications were also collected.

2.2. Chemical calcium extraction

For extraction and quantification of calcium, frozen samples were put in 100 ul HCl 0.6 N, heated at 99° for 5 min and lysed through 2 cycles of freezing and thawing by snap freezing in liquid nitrogen and thawing on ice. Samples were left overnight in HCl 0.6 N before mechanical disruption with TissueLyser (Qiagen). HCl was neutralised with 100 ul of 0.1 N NaOH, 0.1% SDS. Samples were centrifuged at room temperature at 13,000 g. Surnatants were assed for calcium concentration by using O-cresolphtalerin complexome method (Chema Diagnostica, Italy). Protein concentration was assessed by using NanoVue Plus (GE Life Sciences). As quantification of chemically extracted plaque calcium provides the best estimate of plaque calcification, we divided diabetic and non-diabetic patients into 2 equal groups based on the median value of plaque calcium.

2.3. Histologic analysis of carotid plaques

Seven-micrometer thick transversal serial cryosections of each atherosclerotic plaque were stained with Von Kossa and Alizarin red for analysis of calcification, according to the manufacturer's instructions (Bio-Optica Milano Spa). There was a good concordance between the two histological stainings for calcification (VK versus AR $r = 0.64$; $p < 0.001$) and both correlated directly with absolute calcium quantification from plaque digestion (VK: $r = 0.52$; AR: $r = 0.57$; $p < 0.01$ for both). The pentachromic (Movat's) staining was used to characterize atherosclerotic lesion components, according to the manufacturer's instructions (American MasterTech). In this staining, black colouring is for nuclei and elastic fibres, yellow for collagen fibres, blue for mucin, bright red for fibrin and red for muscle. It should be noted that the sum of the individual percentages of these components is >100%, because automated colour analysis detects overlapping patterns and a given area can be considered occupied, e.g., by mucins and collagen at the same time. Hematoxylin and eosin staining was also performed in parallel. Custom ImageJ plug-ins were used for quantification of calcium deposits and tissue composition, which were expressed as percentage of section area.

2.4. Gene expression analysis

Total RNA was extracted using RNeasy kit (Qiagen), then reverse transcribed to generate cDNA using the First-Strand cDNA Synthesis Kit from Invitrogen. Gene-specific primer pairs were designed using Primer-BLAST (NCBI) and were validated prior to use by gradient PCR and gel analysis to test for optimal annealing temperature, reaction efficiency and specificity. Real time PCR with Fast SYBR Green detection was performed using an 7900HT Fast Real-Time qPCR System (Applied Biosystems) and the primers shown in Table S1. Expression data were normalized to the mean of housekeeping gene beta-actin to control the variability in expression levels and were analysed using the $2^{-\Delta\Delta CT}$ method.

2.5. Statistical analysis

Data are expressed as mean ± standard error, or as percentage, where appropriate. Non-normal variables were log-transformed before statistical analysis. Differences between two groups were assessed using the unpaired 2-tail Student's t test for continuous variables, or the chi square test for categorical variables. Differences among more than two groups were checked with ANOVA followed by post-hoc Benjamini–Hochberg correction for false discovery rate. Linear correlations were analysed using the Pearson's r coefficient. Regression coefficients were statistically compared using the Fisher z-transformation. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Patients' characteristics

The study included $n = 59$ patients (age range 54–81 years) enrolled at time of CEA, divided into $n = 23$ diabetic patients and $n = 36$ non-diabetic controls. These 2 groups were further divided according to the presence of low or high plaque calcium content. Clinical characteristics are summarized in Table 1. Except from fasting plasma glucose, HbA1c and glucose-lowering therapy, diabetic and non-diabetic patients were comparable in terms of demographics, prevalence of risk factors, comorbidities, and general cardiovascular medications. This indicates that eventual differences in plaque composition or characteristics should be attributable uniquely to diabetes per se and not to associated factors or confounders. No significant clinical difference was detected among diabetic or non-diabetic patients according to plaque calcium. Carotid plaques were associated with a history of stroke or TIA in 21% of non-diabetic and 26% of diabetic patients.

3.2. Relationships between plaque calcification and histologic composition

We randomly selected a subgroup of $n = 10$ diabetic and $n = 10$ matched non-diabetic patients from the entire population, to perform a detailed histological characterization of plaque composition using the Pentachromic staining. As shown in Fig. 1a,b there were no overall significant differences in the composition of carotid plaques from diabetic versus non-diabetic patients, in terms of mucin, collagen and muscle/fibrinoid tissue.

Plaque calcium content was similar in diabetic versus non-diabetic patients (Fig. 1c). In this subgroup of 20 patients, plaque calcium content was directly correlated with the area occupied by