International Journal of Cardiology 174 (2014) 503–515

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Towards non-invasive imaging of vulnerable atherosclerotic plaques by targeting co-stimulatory molecules

Adrienne Müller [a,*,1], Linjing Mu [b,1], Romana Meletta [a,1], Katharina Beck [a,1], Zoran Rancic [c,1], Konstantin Drandarov [a,1], Philipp A. Kaufmann [d,1], Simon M. Ametamey [a,1], Roger Schibli [a,b,1], Nicole Borel [e,1], Stefanie D. Krämer [a,1]

[a] Department of Chemistry and Applied Biosciences of ETH Zurich, Center for Radiopharmaceutical Sciences ETH-PSI-USZ, Zurich, Switzerland
[b] Department of Nuclear Medicine, University Hospital Zurich, Center for Radiopharmaceutical Sciences ETH-PSI-USZ, Zurich, Switzerland
[c] University Hospital Zurich, Clinic for Cardiovascular Surgery, Zurich, Switzerland
[d] University Hospital Zurich, Department of Nuclear Medicine, Cardiac Imaging, Zurich, Switzerland
[e] Institute for Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

ARTICLE INFO

Article history:
Received 21 November 2013
Received in revised form 3 April 2014
Accepted 4 April 2014
Available online 13 April 2014

Keywords:
Atherosclerosis
Vulnerable plaque
Immune system
Co-stimulatory molecules
Positron emission tomography

ABSTRACT

Background: Myocardial infarction and stroke are the life-threatening consequences after plaque rupture in coronary or carotid arteries. Positron emission tomography employing [^{18}F]fluorodeoxyglucose can visualize plaque inflammation; however, the question remains whether this is specific for plaque vulnerability. The pathophysiology of vulnerable plaques suggests several molecular processes. Here, we propose the co-stimulatory molecules CD80 and CD86 as potential new targets for non-invasive imaging.

Methods and results: Human atherosclerotic segments were obtained from carotid endarterectomy and classified into stable and vulnerable plaques. We identified CD80 and CD86 with significantly higher mRNA levels in vulnerable than stable plaques. CD80+ and CD86+ cells were found in spatial proximity to CD83+ dendritic cells and CD68+ macrophages of atherosclerotic plaques. As a proof of target-expression we labeled a low molecular weight ligand, which has a high affinity for human CD80, with carbon-11 to perform in vitro autoradiography with human plaque slices. We observed 3-fold higher binding to vulnerable than stable plaques, demonstrating a first approach towards discriminating between the two plaque types. Positron emission tomography studies showed accumulation in CD80+ Raji xenografts, low radioactivity in myocardium and rapid clearance from the blood pool in mice.

Conclusion: In human carotid arteries, the co-stimulatory molecules CD80 and CD86 show significantly higher expression levels in vulnerable compared to stable plaques. With the novel CD80-specific radiotracer we are able to discriminate between stable and vulnerable atherosclerotic plaques in vitro. This is an important step towards non-invasive imaging of the life-threatening vulnerable lesions in humans.

1. Introduction

The immune system is a network of cells, tissues and organs that act in concert to defend the body against foreign invaders. It depends on the ability to distinguish between self and non-self molecules. Failure to this may lead to autoimmune diseases. Both the innate and adaptive immune systems participate in the pathogenesis of atherosclerosis and its clinical events. For many years, it was believed that atherosclerosis develops due to passive accumulation of low-density lipoproteins (LDL) in the vessel wall. Today, the picture is more complex, atherosclerosis is thought of as a chronic inflammatory disease sharing many features with other chronic inflammatory disorders such as rheumatoid arthritis, chronic obstructive pulmonary disease (COPD) and further autoimmune diseases [1,2].

Key effector cells of the immune system, such as antigen presenting cells (dendritic cells, DC; B cells) and T cells accumulate in atherosclerotic plaques and determine the disease progression. Since DC are integral components in atherosclerotic lesions of human arteries, numerous studies suggest a role for these professional antigen presenting cells (APC) in atherosclerosis [3,4]. The general steps for T cell activation by APC comprise: first, recognition of the presented antigen by a T cell receptor; second, ligation of the major histocompatibility complex (MHC), expressed on APC, with the T cell ligands CD4 or CD8; and third, interaction of co-stimulatory molecules on the APC with T cell-specific receptors.

* Corresponding author at: Center for Radiopharmaceutical Sciences of ETH-PSI-USZ, Department of Chemistry and Applied Biosciences, ETH Zurich, Vladimir-Prelog-Weg 3, CH-8093 Zurich, Switzerland. Tel.: +41 44 633 60 84; fax: +41 44 633 13 67.
E-mail address: adrienne.mueller@pharma.ethz.ch (A. Müller).
[1] These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

http://dx.doi.org/10.1016/j.ijcard.2014.04.071
0167-5273/

In the context of atherosclerosis, numerous antigens have been suggested including proteins from pathogenic microorganisms such as heat shock protein 60 (HSP60) from *Chlamydia pneumoniae* or self-antigens such as HSP60, LDL or LDL modified by oxidation to oxLDL, and β2-glycoprotein I [5–8]. Co-stimulatory molecules are powerful agents that modulate the immune system towards either pro- or anti-inflammatory responses. In the absence of a co-stimulation or owing to a co-inhibitory signal, the T cell enters into a state of anergy. One of the best characterized groups of co-stimulatory molecules is the B7 family. Members of this family interact as follows: APC ligands CD80 (B7.1) and CD86 (B7.2) are recognized by the T cell receptors CD28 and CTLA4, respectively; ICOSL is recognized by ICOS (inducible co-stimulatory), and PD-L1 and PD-L2 by PD-1 (programmed death-ligand). No human receptors have been identified for B7-H3 and B7-H4 [9]. Pathways that are initiated by CD80/CD86:CD28/CTLA4 interactions play a key role in T cell regulation. These pathways do not only provide positive signals which promote and sustain T cell response, but can also contribute to negative signals, which attenuate an immune response. Accordingly, they can set the course in immune diseases and consequently in atherosclerosis [1]. Since CD80 and CD86 bind to the same T cell receptors they elicit overlapping functions. This is shown by the findings that mice with deficiencies in either CD80 or CD86 have mild phenotypes, whereas mice with combined deficiencies have profound defects in T cell response and exhibit reduced atherosclerotic lesion development [10].

Patients suffering from cardiovascular disease display an increased expression of CD80 and CD86 in blood monocyte-derived DC [11]. Moreover, in the human atherosclerotic plaque CD80 and CD86 were found on M1 macrophages (pro-inflammatory type) and DC, and were associated with plaque inflammation [12–15]. High accumulation of pro-inflammatory cells is, beside angiogenesis, a major determinant of plaque vulnerability. In the nascent plaque, the lipid core gains in size and number of inflammatory cells. Apoptosis is a common feature in this situation and results in structural weakening of the fibrous cap. Disruption of this cap can lead to thrombosis or cholesterol embolization from the lipid core resulting in many of the adverse clinical outcomes such as stroke, myocardial infarction or peripheral artery disease. In contrast, the more frequent stable plaques with a thick fibrous cap, small lipid core and low levels of inflammatory cells can cause luminal stenosis and first clinical symptoms (angina pectoris) but are not immediately life-threatening.

Despite enormous efforts, non-invasive discrimination between vulnerable and stable plaques in human coronary and carotid arteries remains elusive. One major reason for this significant gap in cardiovascular diagnostics and therapeutic monitoring is the lack of a common targetable biomarker for the non-invasive staging of plaque vulnerability in both coronary and carotid arteries. This study was designed to determine whether the co-stimulatory molecules CD80 and CD86 and their common receptors CD28 and CTLA4 are associated with plaque vulnerability in human carotid endarterectomy specimens and could, therefore, be used as imaging markers. We identified CD80 and CD86 as promising biomarkers for plaque vulnerability. As a proof of concept towards CD80 imaging by non-invasive positron emission tomography (PET), we labeled a published small molecule inhibitor of CD80 with carbon-11 [16]. Our novel radiotracer, designated [^{11}C]**7**, was evaluated by in vitro autoradiography with human plaque sections for its potential to discriminate between vulnerable and stable plaques. The tracer was characterized for its pharmacokinetic properties in vitro and its in vivo distribution was analyzed in CD80+ xenograft bearing and immune-competent mice by small animal PET.

2. Methods

2.1. Subjects' characteristics, clinical data, tissue banking

This study was conducted with excised tissue from 30 patients referred to the Clinic for Cardiovascular Surgery, University Hospital of Zurich (Switzerland) for carotid endarterectomy (CEA) between 02/2009 and 10/2011. Written informed consent was obtained from all patients before surgery. Before operation, all patients underwent either duplex ultrasound, computed tomography angiography (CTA), or magnetic resonance brain scans. The technique for carotid endarterectomy was previously described as bifurcation advancement [17,18]. Briefly, the arteria carotis communis, externa and interna were clamped; longitudinal arteriotomy was extended from common to internal and external carotid artery, while a temporary shunt ensured blood supply to the brain. Through meticulous endarterectomy the atheromatous plaque(s) were removed from all three carotid arteries. With creation of a neo-bifurcation the arteriotomy was closed. In some cases, a redundant segment of a normal artery wall (A. thyreoidea superior or A. sternocleidomastoidea) had to be removed. All excised specimens were photographed for macroscopic re-evaluation before transfer within 5 min into RNA*later*® solution (Sigma, St. Louis, USA) and storage at 4 °C until further use.

2.2. Histology and immunohistochemistry

For histology and immunohistochemistry, plaque segments were embedded in paraffin and serial sections of 3 μm were cut. Histological sections were then routinely stained with hematoxylin and eosin (HE), Gomori's blue trichrome and Prussian blue staining for iron-containing hemosiderin from previous intraplaque hemorrhage.

For immunohistochemistry, consecutive sections were labeled with the following primary antibodies: anti-CD3 (1:100, A0452, Dako, Glostrup, Denmark), anti-CD68 (1:100, Clone KP1, M0814, Dako), anti-CD80 (1:100, ab134120, Abcam, Cambridge, UK), anti-CD83 (1:25, ab49324, Abcam), and anti-CD86 (1:100, ab53004, Abcam). Subsequent steps were performed on a Discovery XT instrument (Ventana Medical Systems) with antigen retrieval using Cell Conditioner CC1 (predilute Tris/Borate/EDTA buffer solution, pH 8.0 to 8.5). The primary antibodies were visualized using the detection kit OmniUltraMap anti-Rabbit or anti-Mouse HRP (Ventana). The slides were then counterstained with hematoxylin and Bluing Reagent. By using the antibody diluent instead of the primary antibody a negative control of each section was performed.

Sections were evaluated by microscopy (digital slide scanner Pannoramic 250, Sysmex, Horgen, Switzerland). Ten independent investigators blindly scored the stained sections into weak, moderate, and strong staining intensities with a training table as reference. The results were compiled as percentaged average of the ten scores [18].

2.3. RNA isolation, reverse-transcription reaction and real-time polymerase chain reaction

Human atherosclerotic plaques, xenografts of human Raji cells (Burkitt's lymphoma, DMSZ, Braunschweig, Germany) and human NCI-H69 cells (small cell lung cancer, negative control, DMSZ) were used for total RNA isolation according to the protocols of the Isol-RNA Lysis Reagent (5 PRIME, Gaithersburg, USA) and the bead-milling TissueLyser system (Qiagen, Hilden, Germany). QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) was used to generate cDNA. The following primers (Microsynth, Balgach, Switzerland) were used for the quantitative polymerase chain reaction (qPCR): human Actin, beta (ACTB; GenBank accession no. NM_001101), forward 5′-CATGTACG TTGCTATCCAGGC-3′, reverse 5′-CTCCTTAATGTCACGCACGAT-3′; human CD80 (GenBank accession no. NM_005191), forward 5′-GGGCACATACGAGTGTGTTGT-3′, reverse 5′-TCAGCTTTGACTGATAACGTCAC-3′; human CD86 (GenBank accession no. NM_175862), forward 5′-TATGGGCCGCACAAGTTTTGA-3′, reverse 5′-TGGTGGATGCGAATCATTCCT-3′; human CD28 (GenBank accession no. NM_006139), forward 5′-AAGGGAAACACCTTTGTC CAAG-3′, reverse 5′-GCGGGGAGTCATGTTCATGTA-3′; human CTLA-4 (GenBank accession no. NM_005214), forward 5′-GAACCTCACTATCCAAGGACTGA-3′, reverse 5′-CCTATGCCCA GGTAGTATGGC-3′. Quantitation of CD80, CD86, CD28 and CTLA-4 expression was performed with the DyNAmo™ Flash SYBR® Green qPCR Kit (Thermo Scientific, Runcorn, UK) using a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, USA). The amplification signals were detected in real-time, which permitted accurate quantification of the amounts of the initial RNA template during 40 cycles according to the manufacturer's protocol. All reactions were performed in duplicates and within five independent runs. Quantitative analysis was performed using the SDS Software (v2.4) and a previously described $2^{-\Delta\Delta Ct}$ quantification method [19]. The specificity of the PCR products of each run was determined and verified with the SDS dissociation curve analysis feature.

2.4. Protein isolation and western blotting

For preparation of protein lysate, xenografts (Raji, NCI-H69) were grinded in a mortar under liquid nitrogen. The powder was collected in 500 μL CellLyticTM MT buffer (Sigma, St. Louis, MO, USA) containing Halt™ protease inhibitor (Promega, Madison, WI, USA). Mixtures were homogenized by sonification and centrifuged at 12,000 ×*g* for 10 min at 4 °C. The supernatants were collected and analyzed by western blot. Separation of proteins was performed by 10% SDS-polyacrylamide gel electrophoresis, according to standard protocols (Bio-Rad, Hercules, MA, USA). After SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). The blots were blocked with 5% dried milk powder in Tris-buffered saline-Tween 20 (pH 7.4). They were then processed for immunostaining with a rabbit anti-CD80 antibody (1:500, ab53003, Abcam, Cambridge, UK), rabbit anti-CD86 antibody (1:500, ab53004, Abcam, Cambridge, UK) or an antibody against β-actin (1:8000, A2066, Sigma, St. Louis, MO, USA) at 4 °C overnight. Bound antibodies were visualized with anti-rabbit immunoglobulin G (IgG) secondary antibodies conjugated with horseradish peroxidase diluted to 1:80,000 (A0545, Sigma, St. Louis, MO, USA). The antigen-antibody complexes were detected by enhanced chemiluminescence. Western blots were repeated at least twice to confirm results.