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Induced macrophage activation in live excised atherosclerotic plaque

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ABSTRACT

Atherosclerotic plaques are complex tissues containing many different cell types. Macrophages contribute to inflammation, formation of the necrotic core, and plaque rupture. We examined whether macrophages in plaque can be activated and compared this to monolayer cells. The volume of calcium in the plaque was compared to the level of macrophage activation measured by total neopterin output. Carotid plaque samples were cut into 3 mm sections and cultured for up to 96 h. Live sections were stimulated with interferon-γ, phytohaemagglutinin or phorbol 12-myristate 13-acetate. Macrophage activation and oxidative stress were monitored by total neopterin (oxidized and non-oxidized 7,8-dihydroneopterin) and neopterin levels every 24 h for up to 4 d. The calcium content of two plaques was investigated by spectral imaging. Direct stimulation of macrophages in plaque sections with interferon-γ caused a sustained increase in neopterin ($p = .037$) and total neopterin ($p = .003$). The addition of phorbol 12-myristate 13-acetate to plaque had no significant effect on total neopterin production ($p = .073$) but increased neopterin ($p = .037$) whereas phytohaemagglutinin caused a significant increase in both neopterin and total neopterin ($p = .0279$ and .0168). There was an inverse association ($R^2 = 0.91$) between the volume of calcium and macrophage activation as measured by total neopterin production in stimulated plaque tissue. Resident macrophages within excised carotid plaque activated either directly or indirectly generate the biomarkers 7,8-dihydroneopterin and neopterin. Macrophage activation rather than the oxidative environment is associated with plaque calcification.

1. Introduction

Atherosclerotic plaques are complex tissues made up of a number of different cell types and cellular subsets which form in the arterial wall (Stary et al., 1995). Monocytes, a macrophage precursor cell, infiltrate the arterial wall in early atherosclerosis. Once resident, macrophages promote inflammation through the generation of oxidants and the release of inflammatory cytokines (Biessen and Wouters, 2017). Macrophages continue the progression of an atherosclerotic lesion by taking up toxic oxidized low-density lipoprotein (oxLDL) in an uncontrolled manner, causing foam cell formation and eventual cell necrosis (Kavurma et al., 2017; Howell et al., 2011). The subsequent release of lipids from dying macrophages promotes the formation of a lipid or necrotic core in the arterial wall (Silvestre-Roig et al., 2014; Tabas, 2009; Otsuka et al., 2015). This necrotic core is separated from the blood by a thin fibrous cap. Over time, resident macrophages may release matrix metalloproteinases degrading the fibrous cap (Kong et al., 2005), making the plaque prone to rupture (Gao and Long, 2008). Plaque rupture opens the contents of the necrotic core to the blood clotting factors triggering thrombosis (Owens and Mackman, 2012). The thrombus can then break away from the site of plaque rupture and obstruct small vessels. These infarcts typically occur in the brain or heart and often result in death (Psychogios et al., 2015).

Given the high rate of mortality associated with atherosclerosis, there is a strong emphasis on improving detection and diagnosis of the disease. In recent years, there have been significant improvements in various imaging modalities to detect atherosclerosis (Tarkin et al., 2016), and there has been a shift towards identifying biomarkers of the disease, which would provide a non-invasive means of diagnosing and monitoring atherosclerosis (De Rosa et al., 2011; Soeki and Sata, 2016).

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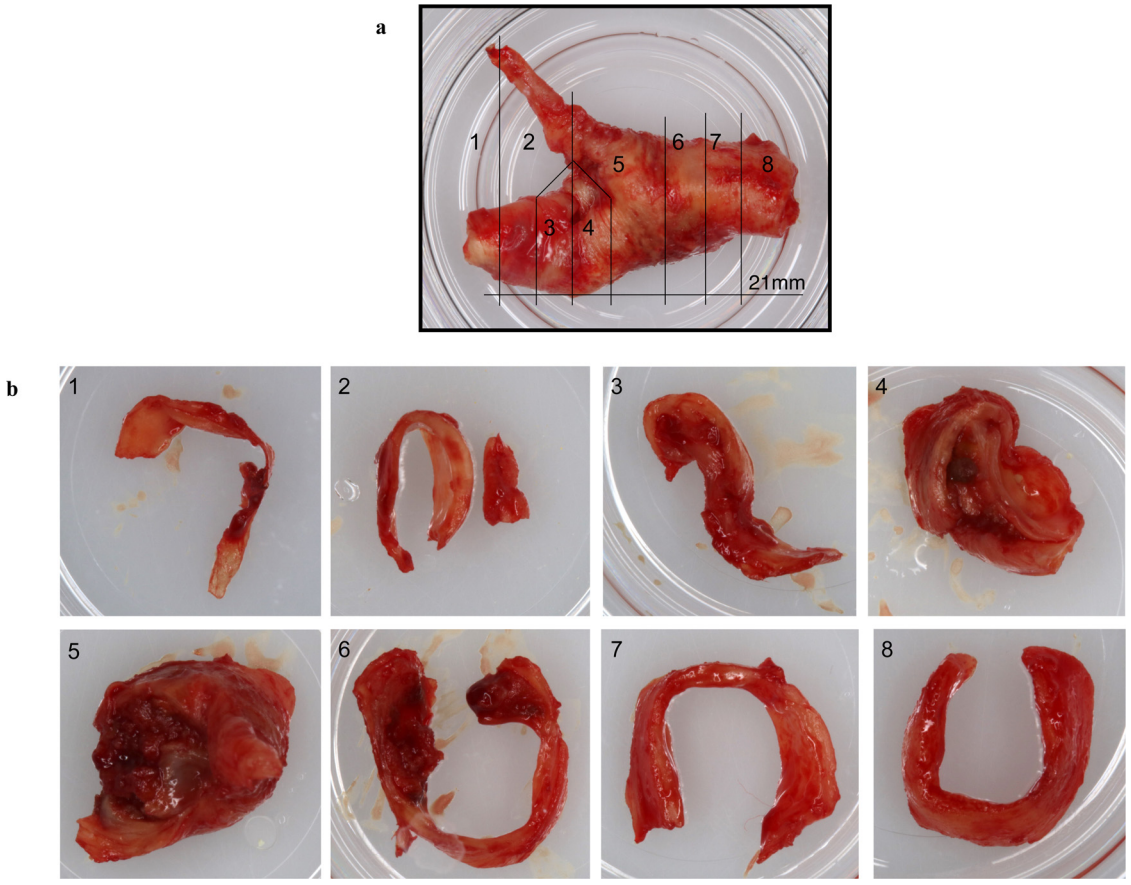

Fig. 1. Example of an Internal Carotid Artery plaque prior to (a) and post sectioning procedure (b). Each section was then placed into an individual organ culture dish for the length of the experiment. The plaque was obtained from a 65-year-old male non-smoker who was suffering from dysphasia and amaurosis fugax. Stenosis of the artery was reported as 80–99%. This plaque was used in the experiments conducted in Fig. 5. All plaque culture experiments were carried out on plaques sectioned in this manner.

Given the key role macrophages play in the development of vulnerable plaque, we have examined the macrophage specific compounds 7,8-dihydroneopterin and its oxidation product neopterin as potential as biomarkers of plaque growth and inflammation. Activated macrophages, those which are displaying an M1 phenotype, produce significant quantities of a guanosine triphosphate (GTP)-derived antioxidant, 7,8-dihydroneopterin, which is oxidized *in vivo* to a highly fluorescent compound, neopterin (Wachter et al., 1989; Gieseg et al., 2001; Gieseg et al., 2008). 7,8-Dihydroneopterin can block cell mediated low density lipoprotein (LDL) oxidation, oxidized LDL induced macrophage death, and down regulate CD36 a key scavenger receptor for oxidized LDL involved in the formation of foam cells (Shchepetkina et al., 2017), a key constituent of plaques. Neopterin has been used clinically to measure immune cell activation in several pathologies including cardiovascular disease (Tang et al., 2016; Ray et al., 2007).

Clinical studies have found that neopterin is elevated in serum of patients with acute ischaemic stroke and myocardial infarction (Elayalwar et al., 2016; Srivastava et al., 2014), and there is prognostic value for patients at risk of ischaemic heart disease or acute coronary syndrome (Vengen et al., 2009; Zeng et al., 2016). To date, there has been little research into the *in vivo* generation of neopterin and most research into the role of neopterin has made use of isolated cultures of macrophages or peripheral blood mononuclear cells (PBMC), however, this approach does not allow for the investigation of the more complex interactions between array of cell types (smooth muscle cells, macrophages, monocytes, T cells) and cytokines within the plaque tissue (Wolf et al., 2015; Hsu et al., 2016). In this study, we overcome this disadvantage of mono layer cultures by activating resident macrophages in live carotid plaque tissue samples from carotid endarterectomy patients. We used three different known macrophage stimulants, interferon-γ, PMA and PHA and compared activation within the plaque tissue with traditional monolayer cultures in the presence and absence of T cells. Interferon-γ directly induces the production of 7,8-dihydroneopterin in macrophages *via* the enzyme GTP cyclohydrolase I (GTPCH-I), while PMA and PHA are known to activate T cells, which produce interferon-γ in response (Schroecksnadel et al., 2011; Jenny et al., 2011; Gostner et al., 2015). Thus, PMA and PHA are capable of indirectly increasing 7,8-dihydroneopterin. As 7,8-dihydroneopterin and its oxidation product neopterin are of low molecular weight, they easily diffuse out of the plaque into the blood stream or tissue culture media and can be measured as serum markers of macrophage activity and oxidation (Gieseg et al., 2008).

Typically, in clinical settings only serum neopterin levels are analysed. In this study, we take this system further by measuring both neopterin and 7,8-dihydroneopterin. Neopterin can be measured directly and 7,8-dihydroneopterin indirectly by artificial oxidation to neopterin. In a sample that has undergone this artificial oxidation, we refer to the measured value as 'total neopterin' as the value given contains both the artificially oxidized 7,8-dihydroneopterin and any biologically produced neopterin (Lindsay et al., 2014; Lindsay et al., 2016). The concentration of 7,8-dihydroneopterin alone can be inferred by subtracting the known neopterin value which was measured in the non-oxidized sample. The advantage to measuring both is that we can measure both the amount of oxidation (as neopterin) and the level of macrophage activation (as total neopterin) occurring in the tissue. In doing this, we found that macrophage activation but not the level of oxidation appears to be associated with the amount of calcification in the plaque tissue.