INTRODUCTION

Coronary artery disease (CAD) is characterized by atherosclerotic changes in the arterial wall [1], and different components of the vascular, metabolic, and immune systems are involved in this process. Most of the previous studies investigating morphology of CAD have been focused on changes in the innermost layer of the arterial wall, namely the tunica intima, however over the last few years, there is a growing interest in the role of the other layers in CAD development [2,3].

The arterial wall consists of three, structurally different, layers: tunica intima (innermost layer), tunica media (medial layer), and tunica adventitia or externa (outer layer). The tunica media is made up of multiple layers of vascular smooth muscle cells (VSMCs), supported by connective tissue. The media is normally avascular, and VSMCs in this compartment have contractile as well as synthetic functions (i.e., maintaining the extracellular matrix [ECM]) [4]. Coronary arteries (CAs) are muscular arteries which means they have a thicker tunica media with more smooth muscle cells, compared to elastic arteries which have a large amount of elastin and collagen fibers in the media. The main role of CAs is to supply the heart muscle with blood.

Inflammatory processes compromise the integrity of the arterial wall, affecting the functions of all three layers. It is assumed that the immune privilege of the media is based on both passive (mechanical) and active (biological) mechanisms. The passive mechanisms include the absence of lymphatic and blood vessels which prevents immune responses, and the presence of elastic lamina as a barrier to leukocyte trafficking [5,6]. The active mechanisms of the tunica media immune privilege involve the synthesis of indoleamine 2,3-dioxygenase (IDO) in VSMCs [7,8] and transforming growth factor beta (TGF-β) [9], low expression of major histocompatibility complex (MHC) molecules [10], absence of costimulatory molecules (e.g., OX40 ligand and inducible T-cell costimulator [ICOS] or CD278) [11], and presence of coinhibitory molecules (e.g., programmed death-ligand [PD-L] 1 and 2) [11]. The
failure of the tunica media immune privilege manifests as an intense infiltration of leukocytes, damage and loss of VSMCs, and the destruction of the ECM architecture [4].

In normal arteries, the media does not contain small blood vessels (vasa vasorum) with endothelial cells that could attract immune cells [4,12]. However, in some circumstances neovascularization of the media may occur, for example, when the intima is abnormally thickened [13]. Studies have shown that, in atherosclerotic CAs, the degree of neovascularization of the intima and media correlates with the intensity of inflammation [14]. In other cases, VSMCs can excrete factors that promote angiogenesis [15,16], affect the function of macrophages and T cells, and enhance/amplify the innate and adaptive immune response [4]. In the adaptive immune response, which plays an important role in atherogenesis, T cells recognize self-antigens and induce a humoral immune response that involves the activation of B cells [17].

Different immune cells have distinct functions in the development of inflammation, remodeling of the ECM and weakening of the tunica media. The aim of our study was to assess the immune privilege status of the media in atherosclerotic CAs by determining the abundance and proportion of macrophages, T and B cells in the tunica media in relation to the other layers of the arterial wall, as well as by evaluating vasa vasorum neovascularization of the media.

MATERIALS AND METHODS

Tissue samples

We obtained tissue samples of 36 symptomatic patients with diffuse CAD (aged 60–72 years), who underwent coronary endarterectomy. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (NMEC 170/07/13, NMEC 110/03/16). All participants provided their written informed consent to participate in this study. The study was conducted according to the Declaration of Helsinki, 2013.

Immediately after the surgery, the samples of CAs were fixed for 24 hours in formaldehyde and embedded in paraffin. The tissue specimens were cut transversely with a microtome into 5 µm-thick serial sections and stained with hematoxylin and eosin (HE). The severity of atherosclerosis was graded according to the modified American Heart Association (AHA) classification [18,19]. Among the 36 samples of CAs, fibrous-cap atheroma was found in 14 CAs (38.9%), thin-cap fibroatheroma in 16 CAs (44.4%), and lesions with healed thrombi in 6 CAs (16.7%).

Detection of immune and endothelial cells

Immunohistochemistry was used for the detection of T cells (anti-CD3, pan-T-cell antigen, Dako, Denmark, 1:400), B cells (anti-CD79, B cell antigen, Dako, Denmark 1:50), macrophages (anti-CD68, macrophage antigen, Dako, Denmark, 1:100), and endothelial cells (anti-human von Willebrand factor [vWF], Dako, Denmark, 1:800).

Morphological analysis and statistics

The morphological analysis was performed on three transverse histological sections for each CA.

The number of immune cells was counted in every CA section and expressed as the average ± standard deviation (SD) and percentage of T and B cells, and macrophages in the tunica intima, media and adventitia. A statistically significant difference in the number of immune cells between the intima, media and adventitia was determined by one-way analysis of variance (ANOVA) followed by Scheffe post hoc analysis (p < 0.05).

In the media of the CAs stained with anti-human vWF, the density of vasa vasorum was evaluated as follows: absence of vessels (degree 0), 1 vessel in the section (degree 1), 2 or 3 vessels in the section (degree 2), and 4 or more vessels in the section (degree 3).

We calculated the frequencies and percentages of CAs in relation to the degrees of vascular density.

RESULTS

Morphological analysis

The morphological analysis of HE-stained CAs revealed significant atherosclerotic changes in all specimens. Diffuse fibroproliferation was observed in the intima (Figure 1A). The elastic laminae were interrupted, and in some cases they were completely absent. Focal or diffuse infiltrations of immune cells in the tunica media were localized mainly around vasa vasorum (Figure 1A-F). The disarrangement, damage and loss of VSMCs was observed, as well as the destruction of the ECM architecture leading to fibrosis.

Immune cells in the intima, media, and adventitia

Immune cells were found in the three arterial-wall layers in all CAs. The immunohistochemistry staining showed 43.3% of immune cells in the tunica intima (average 51.9 cells ± 12.7), 50% in the tunica adventitia (60.3 cells ± 19.0), and 6.7% in the tunica media (8.2 cells ± 3.1).

The most abundant immune cells in the media were T cells (51.1%, average 4.2 cells ± 2.8) [p < 0.001 compared to B cells and macrophages], followed by B cells (23.5%, average 1.9 cells ± 1.5), and macrophages (25.4%, average 2.1 cells ± 1.4). The difference between the numbers of B cells and macrophages in the media was not significant (Figure 1C-E; Figure 2A).

In the tunica adventitia and tunica intima macrophages were significantly more numerous. In the adventitia, there were 44.3%
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The density of vasa vasorum in the tunica media was estimated by examining the vWF-stained sections (Figure 1, Figure 3A-C). All CAs had one or more vasa vasorum in the media. Most of the vasa vasorum were observed in the outer part and only a few in the inner part of the tunica media. In the media, there were 14 CAs (38.9%) with 1 blood vessel (degree 1), 13 CAs (36.1%) with 2 or 3 blood vessels (degree 2), and 9 CAs (25.0%) with more than 4 blood vessels [degree 3] (Figure 2B, Figure 3A-C).

DISCUSSION

The main role of the tunica media is maintaining the stability of the vessel wall. Due to the increased secretion of cytokines and chemokines by VSMCs in disease state, immune cells infiltrate the media leading to the remodeling of the ECM and weakening of the media. Moreover, the occurrence of vasa vasorum with endothelial cells in the media amplifies this effect [4].

The aim of our study was to assess the immune privilege status of the tunica media by determining the abundance and proportion of macrophages, T and B cells within the individual wall layers of atherosclerotic CAs, and by estimating the degree

FIGURE 1. (A) Fibroproliferation in the tunica intima of human atherosclerotic coronary arteries (the thickened tunica intima is between the two arrows, HE, 2.5×, bar = 500 µm); (B) Perivascular infiltration (arrows) around vasa vasorum in the tunica media (HE, 40×, bar = 50 µm); (C) B cells (arrows) and vasa vasorum in the tunica media (anti CD79, 40×, bar = 50 µm); (D) T cells (arrows) and vasa vasorum in the tunica media (anti CD3, 40×, bar = 50 µm); (E) Macrophages (arrows) and vasa vasorum in the tunica media (anti CD68, 40×); (F) Endothelial cells (arrows) of vasa vasorum in the tunica media (anti human von Willebrand factor, 63×, bar = 30 µm).
of *vasa vasorum* neovascularization of the tunica media. The histological analysis of atherosclerotic CAs revealed 52% of the immune cells in the intima, 60% in the adventitia, and 8% in the media. In the media, the immune cells were mainly localized around the *vasa vasorum*. In the intima and adventitia, 40% to 50% of the immune cells were macrophages. In the intima, there were approximately 30% of T cells and slightly above 20% of B cells, while in the adventitia, there were 27% of both T and B cells. More than half of the immune cells in the media were T cells, one quarter of the immune cells were B cells and another quarter were macrophages. Our results are similar to those reported by Hagemeijer et al. [20] who investigated the markers of mononuclear cells (MNC), cytokines and chemokine receptors in human CAs with cardiac allograft vasculopathy (CAV). They detected 5- to 10-fold fewer immune cells in the media compared to the intima or adventitia. In the tunica media, they observed 41% of T cells, 7% of B cells and 8% of macrophages [20].

Passive and active mechanisms of immune privilege have been assumed and/or demonstrated in the media, but not in the intima and adventitia [4]. Those mechanisms protect the media from inflammation and their disruption is typically seen in arteriosclerotic diseases, such as atherosclerosis and transplant vasculopathy [4]. The media of normal and atherosclerotic arteries is generally spared of infiltrating immune cells such as leukocytes, T cells and macrophages, and has only scattered resident leukocytes [4,21-23]. However, in response to inflammation, the resident leukocytes as well as infiltrates of other immune cells in the media may stimulate the production of inflammatory cytokines and chemokines in VSMCs and endothelial cells which in turn recruit additional leukocytes [4]. Moreover, in response to diverse stimuli, microbial infection and cellular injury, VSMCs are able to initiate the transcription of both anti- and pro-inflammatory genes. The produced pro-inflammatory molecules, among others, include cytokines that may activate and recruit macrophages, and adhesive molecules that support leukocyte trafficking [4,24-30]. Our analysis showed that a quarter of immune cells in the media of atherosclerotic CAs were macrophages.

Another study showed that VSMCs treated with interferon (IFN)-γ express very high amounts (perhaps 100-fold that of endothelial cells) of IDO, which is an enzyme involved in the catabolism of tryptophan. As the result of tryptophan degradation, T-cell activation and proliferation is inhibited [7,30]. In the intima and adventitia, IFN-γ is produced by activated T cells; the cytokine diffuses into the media and activates synthesis of IDO in VSMCs [4].
The infiltration of T cells in the intima and adventitia prevents their infiltration in the media [4]. In our study, more than half of immune cells in the media were T cells, but the number of T cells in the intima and adventitia was four times higher than in the media. Nevertheless, the T cells in the intima and adventitia represented only about 30% of the total immune cell population in those layers. We assumed that the number of T cells in the intima and adventitia was too small for the production of sufficient quantities of IFN-γ that could activate IDO synthesis in VSMC. Moreover, we observed the disarrangement, damage and loss of VSMCs. Overall, we speculate that due to the damage and loss of VSMCs in the atherosclerotic CAs, the VSMCs could not produce sufficient quantities of IDO, resulting in an increased infiltration of the media with T cells. Thus, the decreased amount of IDO contributed to the disruption of the active immune privilege of the media [4].

To the best of our knowledge, this is the first study to describe the presence of B cells in the media in non-transplant vasculopathy. Macrophages and T cells have been observed in the intima at all stages of atherosclerosis, whereas B cells were only occasionally found within intimal plaques [17,31,32]. In contrast, a larger number of B cells was found in the adventitia [32-34]. In this study, we demonstrated the presence of B cells as well as a high number of T cells in the media. In the atherosclerotic process, T cells recognize self-antigens and induce humoral immunity driven by B cells [17] which contributes to the breakdown of the active immune privilege in the media.

The CAs in our study had a thickened intima, immune cells in all three layers, and one or more vasa vasorum in the media, mainly arising from the adventitia. Similarly, it was shown that in arteries with wall thickening or other pathological changes, vasa vasorum in the media may arise from the adventitial layer or even from the central lumen [35]. Furthermore, in the inner media of aneurysmal and atherosclerotic thoracic aortas, the neovascularization correlated with increased leukocyte infiltration [36,37]. The same result was shown in atherosclerotic CAs where the neovascularization correlated with increased leukocyte infiltration in the intima-media layer [14]. A pro-inflammatory role for the neovascularization of the media has been indicated in murine models [38,39]. Endothelial cells in the vasa vasorum of the media could contribute to the inflammatory process by attracting immune cells, presenting antigens, and demonstrating immunoregulatory properties [12]. Overall, the presence of vasa vasorum in the media contributes to the breakdown of the passive immune privilege in that compartment [4].

It is generally assumed that the ratio of T and B cells, and macrophages in the media of the vascular wall plays an important role in the development of atherosclerotic processes. Further research is needed to better understand the specific events during atherosclerotic changes of the vascular wall.

CONCLUSION

Our results suggest that, in the progressive state of atherosclerotic CAs that contain lesions with healed thrombi, the immune privilege of the media is disrupted by the infiltration of T and B cells, macrophages and the presence of vasa vasorum.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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