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Local extension of HMGB1 in atherosclerotic lesions of human main cerebral and carotid arteries

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Summary. High mobility group box 1 protein (HMGB1) is a non-histone chromosomal protein which is highly conserved, ubiquitous, and widely distributed. HMGB1 has multiple functions in the nucleus, including the maintenance of nucleosome structure, the regulation of gene transcription, and involvement in DNA recombination. HMBG1 is currently recognized to have a wide range of potential functions and pathological relevance. HMGB1 is released into the extracellular space from necrotic cells and from activated macrophages. HMGB1 binds to the receptor for advanced glycation end products, resulting in the induction of inflammatory cytokines, and to endothelial cell thrombomodulin. HMGB1 neutralization may also reduce the development of atherosclerosis and ameliorate brain infarction.

We investigated the immunolocalization of HMGB1 in atherosclerotic lesions of human cerebral and carotid arteries using a specific antibody, and confirmed the detailed expression and cell type localization using double immunofluorolabeling. In the main cerebral arteries, this anti-HMGB1 antibody intensely immunolabeled both normal morphological vascular smooth muscle cells (VSMCs) within the tunica media and infiltrating VSMCs within the intima of thickened fibrous cap plaques. Endothelial cells were also positive for HMGB1. In carotid plaques, HMGB1-like immunoreactivity (IR) was intense in macrophages, although this IR decreased with increasing cell size. Medium-sized foam cells (50-150 μm) were the most intensely stained. This IR was also observed in the nuclei of foam cells and VSMCs. These findings may provide a basis for understanding the association of HMGB1 with atherosclerotic lesions of the cerebral and carotid arteries, and for constructing strategies to counteract atherosclerosis with anti-HMGB1 antibody.

Key words: HMGB1, Vascular smooth muscle cells, Macrophage, Atherosclerosis, Cartotid artery

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Introduction

High mobility group box 1 protein (HMGB1) is a non-histone chromosomal protein. This protein is highly conserved, ubiquitous, and widely distributed, mainly in the nuclei of all cells in mammalian tissues (Mosevitsky et al., 1989). HMGB1 has multiple functions in the nucleus, including the maintenance of nucleosome structure, the regulation of gene transcription, and involvement in DNA recombination (Bustin, 1999). HMBG1 is currently recognized to have a wide range of potential functions and pathological relevance (Poser and Bosserhoff, 2004; Yamada and Maruyama, 2007; de Souza et al., 2012; Peter and Bobik, 2012). HMGB1 is released into the extracellular space from necrotic cells and from activated macrophages. HMGB1 binds to the receptor for advanced glycation end products, resulting in the activation of nuclear factor (NF)-κB (Stern et al., 2002); it also binds to toll-like receptors TLR-2 and TLR-4 (Park et al., 2004; Yu et al., 2006), and to endothelial cell thrombomodulin (Abeyama et al., 2005).

During the inflammatory process, HMGB1 is actively produced by activated macrophages and is also passively released from damaged and necrotic cells (Yamada and Maruyama, 2007). Released HMGB1 induces various inflammatory cytokines in various target organs and tissue sites. Similarly, interleukin-6 (IL-6) is released from carotid plaques after carotid artery stenting (Abe et al., 2010) and may be associated with new ischemic lesions in the brain. Because HMGB1 produced by inflammation may further exacerbate inflammation not only in the original site of inflammation but also in other distant sites of inflammation, it is plausible that HMGB1 not only plays an important role in atherogenesis in carotid and cerebral arteries, but also is released from carotid plaque (mainly by activated macrophages or infiltrating vascular smooth muscle cells [VSMCs]) into the extracellular matrix, then into the lumina of carotid arteries, and finally HMGB1 migration to the brain exacerbates brain inflammation. As a first step to test this hypothesis, we initially investigated the immunolocalization of HMGB1 in atherosclerotic lesions of human cerebral and carotid arteries using a specific antibody, and preliminarily confirmed the cell type localization by double immunofluorolabeling.

HMGB1 neutralization reduces the development of diet-induced atherosclerosis in apolipoprotein E-deficient mice (Kanellakis et al., 2011) and ameliorates brain infarction induced by 2-hour occlusion of the middle cerebral artery in rats (Zhang et al., 2011). As a basis to translate this promising therapeutic attempt in animal experiments into clinical trial, it is necessary to clarify the pathologic features of HMGB1 in cerebral arteries with or without atherosclerosis. To the best of our knowledge, this is the first study to demonstrate the detailed localization of HMGB1 in human cerebral arteries.

Previous reports have demonstrated the expression of HMGB1 not only in macrophages, but also in both spindle-shaped and foamy round VSMCs, although HMGB1-like immunoreactivity (IR) was "undetected" in most nuclei of normal medial VSMCs in normal human arteries (Degryse et al., 2001; Kalinina et al., 2004; Porto et al., 2006; Inoue et al., 2007). HMGB1 stimulates the migration of rat smooth muscle cells (SMCs). HMGB1 acts as a strong chemotactic agent for SMCs (Degryse et al., 2001; Degryse and de Virgilio, 2003), triggering migration from the tunica media to the tunica intima. We succeeded in demonstrating the expression of HMGB1 in normal medial VSMCs in normal cerebral arteries. It has also been reported that HMGB1 expression was not detectable in atrophic VSMCs of fibrous plaques (Degryse et al., 2001; Inoue et al., 2007; Kalinina et al., 2004; Porto et al., 2006). We demonstrated, however, that HMGB1 was retained in thin-shaped VSMCs within fibrous plaques. These results might contribute to the knowledge base determining the indications for HMGB1 neutralization therapy.

Materials and methods

Specimens of carotid arteries were obtained from 18 patients (age range, 56-82 years) undergoing carotid endarterectomy (CEA). Specimens of main cerebral arteries with atherosclerotic changes were obtained from 8 human patients at autopsy (49-93 years old at the time of death). Specimens with minimal atherosclerotic changes were also obtained from 3 human patients at autopsy (63-72 years old at the time of death). Tissue sections (5 μm thick) were prepared from formalin-fixed, paraffin-embedded blocks. We performed hematoxylin-eosin (HE), elastica-van Gieson (EVG) and Masson trichrome (MT) staining.

In this study, atherosclerotic plaques were classified into 3 types (Umahara et al., 2011, 2012) according to the American Heart Association (AHA) classification (Stary et al., 1995). AHA type IV is a thin fibrous cap plaque with a large lipid-rich necrotic core (carotid artery, n=4; cerebral artery, n=0). AHA type V is a thickened fibrous cap plaque with a lipid-rich necrotic core (carotid artery, n=6; cerebral artery, n=3). AHA type Vc is a thickened fibrous cap plaque without a lipid-rich necrotic core (carotid artery, n=8; cerebral artery, n=5).

Deparaffinized sections placed in the citrate buffer were heated in a pressure cooker for 20 min. They were treated with 1% hydrogen peroxide for 30 min. They were then incubated with the primary antibody produced in rabbit against human HMGB1 (1:2000, KPDAAKKGVVKAEK; Shino-test, Kanagawa, Japan) at 4°C for 2 days. The sections were then incubated with the appropriate biotinylated secondary antibody for 2 hours. After incubation with the avidin-biotin-peroxidase complex (1:1000, ABC Elite; Vector, Burlingame, CA, USA) for 1 hour, peroxidase labeling was visualized with a mixture of 0.03% 3,3'-diaminobenzidine, 0.6% nickel ammonium sulfate, 0.05 M imidazole and 0.00015% hydrogen peroxide. A brown (3,3'-diaminobenzidine only) or a deep purple (a mixture of 3,3-diaminobenzidine and nickel ammonium sulfate) immunoreaction product appeared after 15-20 min.

For immunofluorolabeling, after treatment with 0.5% normal goat and horse sera, we incubated deparaffinized sections with a mixture of the anti-HMGB1 antibody (1:200), and either anti-human α-smooth muscle actin (α-SMA, 1:100, mouse monoclonal antibody clone 1A4; Dako, Glostrup, Denmark) or anti-human CD68 (1:100, mouse monoclonal antibody clone KP1; Dako), 48 hours. These antibodies were then visualized with a mixture of anti-mouse IgG antibody made in goat serum conjugated with Alexa 488 (1:200; Molecular Probes, Eugene, OR, USA), and anti-rabbit IgG made in goat serum conjugated with Alexa 546 (1:200; Molecular Probes) for 2 hours. They were then incubated with 4',6-diamino-2phenylindole (DAPI). The fluorolabeled sections were observed under a fluorescence microscope equipped with a laser confocal system (Olympus FV1000; Olympus Medical Systems,

Tokyo, Japan).

Results

1 Conventional immunohistochemistry

1.1 Normal cerebral arteries

In the normal cerebral arteries of all control subjects, where atherosclerotic lesions were not found on HE or EVG staining, HMGB1-like IR was observed in VSMCs of the tunica media (Fig. 1A, arrows) and vascular endothelial cells (Fig. 1A, arrowheads). Approximately 0 to 20% of the nuclei of the VSMCs in the tunica media were intensely positive for HMGB1, although the positive ratios of these varied in each of the sections. In contrast, the cytoplasm of the VSMCs in the tunica media was only slightly immunolabeled with this antibody (Fig. 1A). The location of the HMGB1-positive cells was not consistent. There were few or no CD68-positive cells in the normal cerebral arteries.

1.2 Atherosclerotic lesions of main cerebral arteries

In the atherosclerotic lesions of the main cerebral artery, HMGB1-like IR was intense in the nuclei of the infiltrating cells within the thickened tunica intima (Fig. 1B, arrow), and in the morphologically normal tunica media (Fig. 1B, asterisk) similar to the control subjects. The cytoplasm of the infiltrating cells (Fig. 1B) within the thickened tunica intima was also positive for HMGB1.

1.3 Carotid plaques

In the carotid artery lesions of the CEA specimens, HMGB1-like IR was observed in many foam cells located in the periphery of lipid-rich necrotic cores (Fig. 1C, arrow), in multinucleated giant cells (Fig. 1D, arrow), located mainly within the thin fibrous cap plaque, and in various cells with diverse morphologies mostly found within the thickened fibrous cap plaque. Medium-sized foam cells (50-150 μm) were the most intensely labeled. However, the necrotic cores, fibrous caps, and extracellular matrices, including fibrin-rich lesions of the tunica intima, were negative or only weakly positive for HMGB1 (Fig. 1C, asterisk).

2 Immunofluorolabeling

2.1 Normal cerebral arteries

Double immunofluorolabeling for HMGB1 and α-SMA showed that approximately 5 to 20% of the nuclei of the VSMCs, which were positive for α-SMA within the tunica media, were intensely positive for HMGB1 (data not shown). In contrast, the cytoplasm of the VSMCs within the tunica media was only weakly immunolabeled with this antibody. A few CD68-positive cells were observed within the tunica media and intima, and the cytoplasm of some of these cells was positive for HMGB1 (data not shown).

2.2.a. α-SMA–positive cells in atherosclerotic lesions of main cerebral arteries

In the middle cerebral arteries with atherosclerosis, this anti-HMGB1 antibody partially immunolabeled the infiltrating VSMCs within the intima of thickened fibrous cap plaques (data not shown). Endothelial cells were also positive for HMGB1 (data not shown). In 5 out of 8 main cerebral arteries, the number of nuclei intensely positive for HMGB1 was relatively high in the VSMCs near the shoulder of the thickened tunica intima. The immunolabeling pattern of the cytoplasm of the VSMCs was not significantly different between areas near to and distant from plaque lesions (data not shown).

2.2.b. CD68-positive cells in atherosclerotic lesions of main cerebral arteries

A small number of CD68-positive cells were observed within the thick tunica intima, and the cytoplasm of most of these cells was positive for HMGB1 (data not shown). Few or no CD68-positive cells were observed within the tunica media (data not shown).

2.3.a. α-SMA–positive cells in carotid plaques

HMGB1-like IR was limited in the infiltrating VSMCs within the carotid plaques (Fig. 2B,C). In all cases, at least a small number of HMGB1-positive VSMCs was observed. In each individual case, the number and location of HMGB1-positive VSMCs varied. The positive ratio for HMGB1 in VSMCs was 10 to 80%, although the positive ratios for HMGB1 in VSMCs were not different among each AHA plaque type. The number of HMGB1-positive VSMCs was not related to the number of HMGB1/CD68-positive cells, which include macrophages, foam cells and multinucleated giant cells. HMGB1-positive VSMCs tended to be clustered, and in these clustered areas, more than 70% of the VSMCs were positive for HMGB1. In thickened fibrous plaques (AHA type Vc), both foamed and thin-shaped VSMCs were positive for HMGB1 (Fig. 2B,C), although foamed VSMCs were few in this plaque type (Fig. 2A). In contrast, surrounding the HMGB1-positive cells, clusters of mostly HMGB1-negative VSMCs were observed (Fig. 2D).

Within the tunica media of specimens obtained by CEA, many VSMCs were positive for HMGB1 (Fig. 2E, asterisk). In contrast, within the adjacent thickened intima, which is separated from the tunica media by an internal elastic lamina, only scattered HMGB1-positive cells were observed.

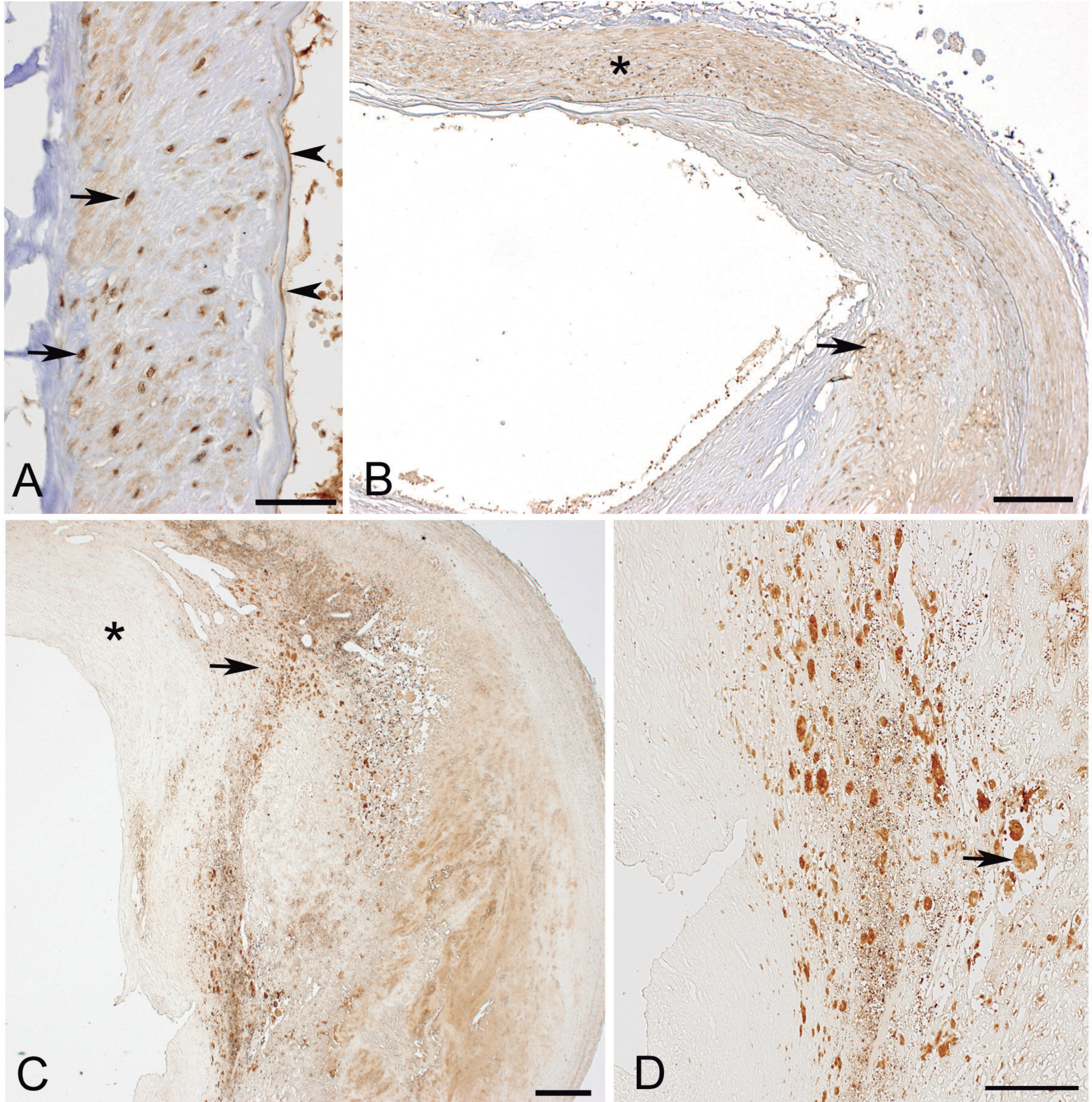

Fig. 1. Immunohistochemical labeling of HMGB1 in basilar and carotid arteries. **A.** Normal basilar artery. HMGB1-like immunoreactivity (IR) was observed in vascular smooth muscle cell (VSMC) nuclei (arrows) in morphologically normal tunica media. The cytoplasm of VSMCs was weakly positive for HMGB1. Vascular endothelial cells were also immunolabeled with this antibody (arrowheads). **B.** Atherosclerotic lesion of the basilar artery. HMGB1-like IR was observed in the infiltrating cells (arrow) in the thickened intima. Tunica media VSMCs (asterisk) were also positive, similarly to morphologically normal tunica media **(A)**. **C.** Carotid artery lesion specimen obtained by carotid endarterectomy (CEA). HMGB1-like IR in many foam cells (arrow) in CEA specimens was observed. Fibrous caps (asterisk) and extracellular matrices of the tunica intima were not positive for HMGB1. **D.** Magnified image of C. At the bottom of a fibrous-cap tear (arrow), a cluster of HMGB1-positive cells was located. Multinucleated giant cells were weakly positive for HMGB1 (arrow). Bars: A, 100 μm; B, D, 500 μm; C, 1 μm.

Fig. 2. Double immunofluorolabeling of HMGB1 (green areas) and α-smooth muscle actin (α-SMA) (red areas) in a carotid plaque. **A.** α-SMA–positive spindle-shaped and thick VSMCs in a thick fibrous cap plaque. **B.** Merged image (yellow) of αSMA (red area, **A**) and HMGB1 (green area) in a fibrous plaque. HMGB1-IR was observed in the nuclei and cytoplasm of both spindle-shaped and thick VSMCs. **C.** HMGB1-positive spindle-shaped and thick cells. **D.** HMGB1-negative VSMCs near HMGB1-positive foam cells (merged image of HMGB1, green and α-SMA, red). **E.** Remaining tunica media specimen obtained by CEA (merged image). Many VSMCs (asterisk) positive for HMGB1 in the tunica media and in the adjacent thickened intima, which is separated from the tunica media by an internal elastic lamina, only scattered HMGB1-positive cells were observed. Bars: A-C, E, 100 μm; D, 500 μm.

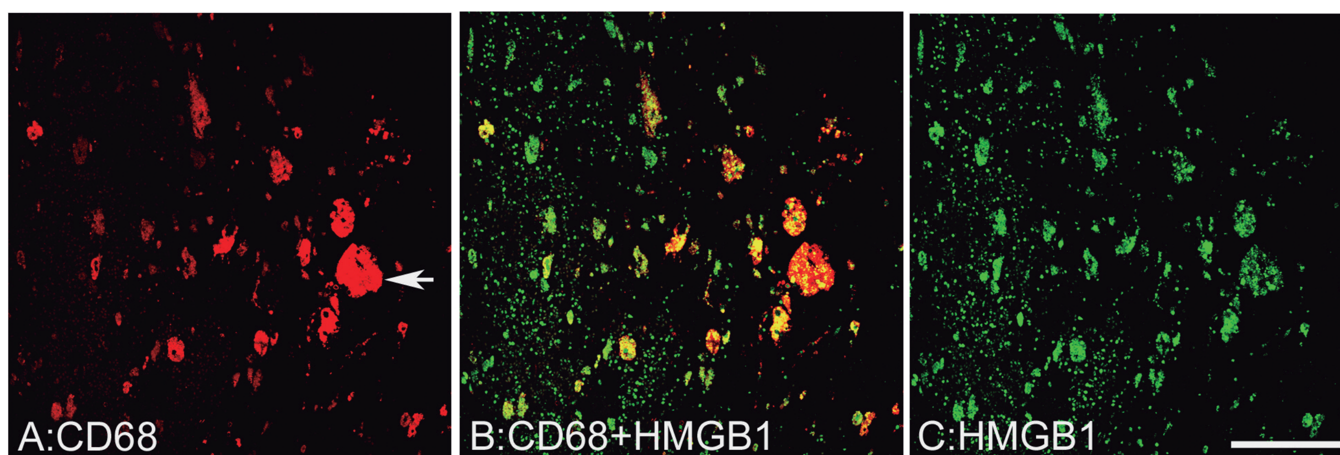

Fig. 3. Double immunofluorolabeling of the CD68 (**A**, KP1, red areas) and HMGB1 (**C**, green areas) in the thin fibrous cap plaque of carotid arteries. A merged image of HMGB1 and CD68 is shown in image **(B)**. Many foam cells and multinucleated giant cells were positive for both CD68 and HMGB1 (**B**, yellow area). However, the gradation of each intensity was contrastive. CD68-like IR increased with increasing cell size. Giant cells were strongly immunolabeled with anti-CD68 antibody (arrow in 3A). In contrast, HMGB1-like IR decreased with increasing cell size. HMGB1-like IR was most intense in mainly medium-sized foam cells (50-150 μm). Bar: 500 μm.

2.3.b. CD68-positive cells in carotid plaques

Many foam cells and multinucleated giant cells were positive for both HMGB1 and CD68 within the thin fibrous cap plaques of carotid arteries (Fig. 3). However, immunoreactivities for these epitopes were not in parallel. CD68-like IR increased with increasing cell size. Giant cells were strongly immunolabeled with the anti-CD68 antibody (Fig. 3A, arrow). In contrast, HMGB1-like IR decreased with increasing cell size. HMGB1-like IR was most intense in mainly medium-sized foam cells (50-150 μm) (Fig. 3). This IR was not restricted to nuclei and was extended into the cytoplasm with uneven distribution as seen in many multinucleated giant cells located mainly within the thin fibrous cap plaques (Fig. 3C).

Discussion

In atherosclerotic lesions, 2 types of cells, namely, macrophages, which are positive for CD68, and SMCs, which are positive for α-SMA (Schwartz et al., 2000; Askari et al., 2002; Umahara et al., 2012), infiltrate the neo-intima and play important roles in atherogenesis. During atherogenesis macrophages take up lipids (Umahara et al., 2011) and are then transformed into foam cells, while differentiated VSMCs in the contractile phase undergo phenotypic changes to an immature form in the synthetic phase.

Both cell types (i.e., macrophages and VSMCs) were positive for HMGB1 in previous studies (Degryse et al., 2001; Degryse and de Virgilio, 2003; Inoue et al., 2007; Kalinina et al., 2004; Porto et al., 2006) and in the present study. However, to the best of our knowledge, this is the first study that distinguished the immunolocalization of HMGB1 in relation to detailed cellular and subcellular structures involved in atherosclerotic lesions in human cerebral and carotid arteries. Although the localization of HMGB1 in the aorta (Kalinina et al., 2004) and coronary artery was well documented, and that in the carotid artery was also reported (Porto et al., 2006; Inoue et al., 2007), our results were partially different from these previous reports. The different pattern of plaque types between the main cerebral artery and the carotid artery may be due to fundamental pathological differences. Because we used specimens of the main cerebral arteries with atherosclerotic changes obtained from cadavers at autopsy, the atherosclerotic lesions that we examined were relatively chronic and benign lesions (Labadzhyan et al., 2011; Umahara et al., 2012) and VSMCs were more predominant than macrophages.

Localization of HMGB1 in normal cerebral arteries

Although HMGB1 is a ubiquitous nuclear protein, previous reports stated that the nuclei of VSMCs on normal appearance were either negative or only faintly positive for HMGB1 (Degryse et al., 2001; Inoue et al., 2007) or that HMGB1 expression in VSMCs was mostly localized close to the endothelium (Kalinina et al., 2004). In this study, we clearly demonstrated the expression of HMGB1 in the nuclei of VSMCs in normally appearing human cerebral arteries. Approximately 0 to 20% of the nuclei of the VSMCs within the tunica media were intensely positive for HMGB1.

As few or no macrophages were present in normally appearing arteries, it is unlikely that HMGB1 is provided by the macrophages to the VSMCs. Vascular endothelial cells were also immunolabeled with this antibody. Although production of HMGB1 may be confirmed by demonstrating its mRNA by in situ hybridization, the presence of HMGB1 immunoreactivity in vascular endothelial cells may be compatible with the hypothesis that HMGB1 is taken up by endothelial cells from circulation. Because HMGB1-positive VSMCs is not situated in close proximity to HMGB1-positive endothelial cells, separated by internal elastic lamina, it is likely that VSMCs might express a small amount of HMGB1 in this condition as well. Indeed, Porto and colleagues (Porto et al., 2006) stated that VSMCs are not only the targets but also the sources of HMGB1. HMGB1 might maintain the nuclei of VSMCs in good structural and functional condition, because HMGB1 was mainly positive in the nuclei of these VSMCs.

VSMCs and HMGB1 localization in atherosclerotic lesions

HMGB1 acts as a strong chemotactic agent for VSMCs (Degryse et al., 2001; Degryse and de Virgilio, 2003), triggering their migration from the tunica media to the tunica intima. Two different pathways are possible candidates for producing and supplying HMGB1 for this migration. One mechanism might be that certain signals stimulate HMGB1 expression by the VSMCs in the tunica media and then the VSMCs migrate to the tunica intima from the tunica media. The other mechanism might be that the VSMCs in the tunica media take up HMGB1 from the blood or from HMGB1 expressed by other cells. Our results may support the former mechanism.

It is still unclear whether VSMC accumulation in the tunica intima is considered "beneficial or harmful in atherogenesis" (Schwartz et al., 2000; Askari et al., 2002). Inoue and colleagues (Inoue et al., 2007) reported that HMGB1 directly stimulates the production of C-reactive protein and matrix metalloproteinase, which is produced by activated VSMCs and may contribute to the vulnerability and progression of atherosclerotic lesions toward rupture. In contrast, the formation of a fibrous structure made up of intimal VSMCs could encapsulate the necrotic core and therefore contribute to a beneficial healing response (Schwartz et al., 2000). Increasing age was associated with a decrease in the amount of VSMCs (van Lammeren et al., 2011). Whether VSMC accumulation is beneficial or harmful may depend on the

stage of the atherosclerotic lesion (Askari et al., 2002) and on the age of the person.

Unfortunately, we could not demonstrate the relationships between HMGB1 expression or retention in the VSMCs and stage of atherosclerosis. HMGB1 was still retained in thin VSMCs within thickened fibrous cap plaques (AHA type V) (Fig. 2). However, HMGB1-negative VSMCs accumulated around HMGB1-positive foam cells (Fig. 2). This finding suggests that VSMCs do not always take up HMGB1 from macrophages passively.

Anti-HMGB1 antibody therapy might inhibit HMGB1 expression or suppress its functions in activated VSMCs, resulting in the prevention of plaque rupture. However, this therapy might also inhibit the migration of VSMCs to the tunica intima from the tunica media. It is therefore possible that its efficacy may be influenced by the stage of atherosclerosis and the age of the patient.

CD68-positive foam cells and HMGB1 localization

The amount of HMGB1 expression may change according to macrophage size. So-called “foam cells” (50 to 150 μm) may have the maximum content of HMGB1. Macrophages take up lipids in the tunica intima and become “foamed” and the foam cells release inflammatory cytokines. HMGB1 is released into the extracellular space from activated macrophages and the nuclei of necrotic cells. In carotid plaques, our results suggest that activated macrophages exhibited more intense HMGB1-like IR than necrotic cell nuclei. Giant macrophages, which take up more lipid and undergo cytolysis, retain less HMGB1. The necrotic core, which results from the cytolysis of lipid-rich cells, was either negative or only weakly positive for HMGB1. We recently reported the potential detection capability of macrophage infiltration in human thick fibrous carotid plaques (AHA V) using 3D tomographic ultrasound imaging (Koyama et al., 2013). Both thin and thick fibrous cap plaques might be good targets for anti-HMGB1 antibody therapy.

Potential remote effects to the brain

Because HMGB1 induces IL-6 (Andersson et al., 2000; Agnello et al., 2002; Inoue et al., 2007; Abe et al., 2010) and HMGB1 is up-regulated in macrophages and infiltrated VSMCs within human atherosclerotic lesions, it is expected that IL-6 is up-regulated in human atherosclerotic lesions. Indeed, Abe and colleagues (Abe et al., 2010) reported that IL-6 is released from carotid plaques after stenting and may be associated with new ischemic lesions in the brain. Down-regulating HMGB1 in carotid plaques could reduce IL-6 expression in carotid plaques, resulting in the prevention of secondary ischemic insults to the brain.

Moreover, the HMGB1 expressed in carotid plaques might be conveyed to the brain and stimulate inflammatory cytokines in brain tissue. Yamada and Maruyama (2007) proposed remote mechanisms of organ dysfunction induced by HMGB1which is secreted by the nuclei of necrotic cells and activated macrophages. Medium-sized foam cells, which are present around the necrotic core of carotid plaques, may be a potential source of HMGB1.

In conclusion, we demonstrated the expression of HMGB1 in human main cerebral arteries as well as in carotid and main cerebral artery atherosclerotic lesions. Medium-sized macrophages were strongly positive for HMGB1. HMGB1 retention in VSMCs was varied. At least at some stage, HMGB1 may play an important role in the local migration of VSMCs from the tunica media to the tunica intima and in the thickening of the cap by VSMCs. From our results, effective treatment of carotid atherosclerosis by HMGB1 neutralization might be importantly dependent upon the plaque development stage.

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