Journal of the Neurological Sciences 317 (2012) 106–111

Contents lists available at SciVerse ScienceDirect

Journal of the Neurological Sciences

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

Isoform-specific immunolocalization of 14-3-3 proteins in atherosclerotic lesions of human carotid and main cerebral arteries

Takahiko Umahara [a,c,*], Toshiki Uchihara [c], Shunichi Koyama [a], Takao Hashimoto [b], Jiro Akimoto [b], Jo Haraoka [b], Toshihiko Iwamoto [a]

[a] Department of Geriatric Medicine, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
[b] Department of Neurosurgery, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
[c] Laboratory of Structural Neuropathology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

ARTICLE INFO

Article history:
Received 15 July 2011
Received in revised form 27 January 2012
Accepted 17 February 2012
Available online 9 March 2012

Keywords:
14-3-3 proteins
Arteriosclerosis
Atherosclerosis
Cerebral main artery
Carotid artery
Endothelial cells

ABSTRACT

14-3-3 proteins are now recognized to have a wide range of potential functions and pathological relevance, such as regulating the intercellular signal processes of differentiation, the development and growth of cells, or preventing or mediating cell apoptosis and survival by controlling the localization of potential signaling molecules. We investigated the immunolocalization of 14-3-3 proteins in atherosclerotic lesions of human cerebral and carotid arteries using 14-3-3 isoform-specific antibodies to distinguish 7 isoforms, and confirmed the cell type localization using double immunofluorolabeling. 14-3-3 common (COM)-like immunoreactivity (IR) was intense, mainly in the foam cells and multinucleated giant cells of the carotid artery. The beta, gamma, epsilon, tau, eta, and zeta (6/7) isoform-specific antibodies showed similar results to those with anti-14-3-3 COM antibody. However, among these isoform-specific antibodies, the anti-eta isoform antibody most intensely immunolabeled multinucleated giant cells and foam cells, and the anti-zeta isoform antibody most intensely immunolabeled infiltrating vascular smooth muscle cells (VSMCs), in carotid plaques. Zeta IR was also observed in one part of the mural thrombus and in the nuclei of foam cells. Gamma isoform-like IR was relatively limited in cell components, but extracellular lesions were partly positive for this isoform. In the main cerebral arteries, the anti-epsilon isoform antibody most intensely immunolabeled infiltrating VSMCs in the intima of thickened fibrous cap plaques. Endothelial cells were also positive for the epsilon isoform. These findings may provide a basis for understanding the isoform-specific role associated with atherosclerotic lesions of the cerebral and carotid arteries.

1. Introduction

In the course of investigating 14-3-3 protein immunolocalization in cerebral infarctions, we observed that some parts of cerebral arteriosclerotic lesions were immunopositive for several 14-3-3 protein isoforms [1]. Thus, we hypothesized that 14-3-3 proteins may play an important role in the development of cerebral arteriosclerosis, which is the cause of different types of ischemic stroke [2].

14-3-3 proteins belong to a family of highly conserved 30 kDa molecules [3,4] and are now recognized to have a wide range of potential functions and pathological relevance [3,5–12]. 14-3-3 proteins regulate the intercellular signal processes of differentiation, development, and growth, and bind to many important target molecules, including transcription cofactors. In addition, they can prevent or mediate apoptosis and survival by controlling the localization of potential signaling molecules between the nucleus or mitochondrion and the cytoplasm.

To date, 7 14-3-3 protein isoforms (β: beta, γ: gamma, ε: epsilon, ζ: zeta, η: eta, τ: tau, and σ: sigma) have been identified in mammals, and each isoform apparently differs in its pathological relevance in human brains [13–15]. The possible effects of several 14-3-3 protein isoforms on vascular injury and thrombosis have been attracting increasing attention. For example, the induction of gamma isoform expression in injured arteries and their associated vascular smooth muscle cells (VSMCs) [16], and the zeta isoform, a granule protein of human platelet dense granules [17] located in atherosclerotic plaques of the abdominal aorta, has been previously investigated [18]. The epsilon isoform is associated with vascular endothelial cell survival [19]. However, detailed data on 14-3-3 protein isoforms associated with vascular biology remain limited. The cellular, subcellular, and extracellular immunolocalizations of each 14-3-3 protein isoform in atherosclerotic plaques of human carotid and cerebral arteries have not yet been investigated. It is important to clarify in detail the distribution of all 7 isoforms in human lesions as a preliminary analysis to identify a therapeutic

* Corresponding author at: Department of Geriatric Medicine, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan. Tel.: +81 3 3342 6111; fax: +81 3 3342 2305.
E-mail address: takahiko@tokyo-med.ac.jp (T. Umahara).

0022-510X/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jns.2012.02.015

target for preventing atherosclerosis and ischemic stroke, because each isoform has a specific function under various conditions (see Table 1).

2. Methods

Specimens of carotid arteries were obtained from 14 patients (age range, 56–82 years) undergoing carotid endarterectomy (CEA). Specimens of main cerebral arteries with atherosclerotic changes were obtained from 8 autopsied human patients (49–93 years old at the time of death). Specimens with minimum atherosclerotic changes were also obtained from 3 autopsied human patients (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 [20] according to the American Heart Association (AHA) classification [21]. AHA Type IV: thin fibrous cap plaque with a large lipid-rich necrotic core and a thin fibrous cap with macrophage infiltration [carotid artery (n = 4); basilar artery (n = 0)]. AHA Type V: thickened fibrous cap plaque with a lipid-rich necrotic core [carotid artery (n = 5); basilar artery (n = 3)]. AHA Type Vc: homogenous fibrous plaque [carotid artery (n = 5); basilar artery (n = 5)] with a thickened fibrous intima and no lipid-rich necrotic core. After heating in citrate buffer in a pressure cooker for 20 min, sections were treated with 1% hydrogen peroxide for 30 min. They were then incubated with one of the primary antibodies produced in rabbits (Immuno-Biological Laboratories, Gunma, Japan) against the human 14-3-3 protein (1:2000, anti-14-3-3 common [COM], KDSTLIMQLLRDNLT, a target sequence shared by all of the isoforms) or each isoform (anti-beta: MTMDKSELVQ,1:500; anti-gamma: QQDDDGGEGNN, 1:200; anti-epsilon: MGDREQLLQR, 1:500; anti-zeta: MDKNELVQK, 1:200; anti-sigma (C): EEGGEAPQEPQS, 1:300; and anti-eta: MGDREQLLQR isoforms), and we used anti-14-3-3 tau isoform monoclonal antibody (raised against the recombinant human 14-3-3 tau isoform) at 4 °C for 2 days. The specificity of these antibodies was established previously on Western blot [13,14,22] and absorption studies [13,14]. There was slight or no cross-reaction among isoform antibodies.

The sections were then incubated with the appropriate biotinylated secondary antibody for 2 h. After incubation with the avidin–biotin–peroxidase complex (1:1000, ABC Elite, Vector, Burlingame, CA, USA) for 1 h, 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 deep purple immunoreaction product appeared after 15–20 min. Positive and negative controls were also incubated.

2.1. Immunofluorolabeling

After treatment with 0.5% normal goat and horse sera, we incubated deparaffinized sections with a mixture of an anti-eta, anti-zeta, or anti-epsilon isoform (1:20), 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) for 48 h. 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-mouse IgG made in goat serum conjugated with Alexa 546 (1:200; Molecular Probes) for 2 h. The fluorolabeled sections were observed under a fluorescence microscope equipped with a laser confocal system (Olympus FV1000, Olympus Medical Systems, Tokyo, Japan).

2.2. Semiquantitative analysis

One hundred CD68- or α-SMA-positive cells in all carotid plaques and 50 cells in all cerebral artery plaques were observed concentrically from the point with the greatest cell accumulation, and the immunopositive ratio for each isoform was analyzed. In the zeta isoform, each positive-nuclear ratio was also evaluated.

The widest area of matrix components in each plaque was measured using Image J (provided by the National Institutes of Health), and the immunopositive-area ratio for each isoform to the total matrix component area was analyzed. Each mean ratio was classified into 5 groups (i.e., 1: 0–20%, 2: 21–40%, 3: 41–60%, 4: 61–80%, and 5: 81–100%).

3. Results

3.1. 14-3-3 COM

In the normal tunica intima of the cerebral arteries of control subjects and CEA specimens, where atherosclerotic lesions were not found on HE or EVG staining, 14-3-3 COM-like immunoreactivity (IR) was absent or faint (unpublished data). In normal tunica media, faint 14-3-3 COM-like IR was limited to some VSMCs (unpublished data; see similar findings in Fig. 1A).

In the atherosclerotic lesions of the main cerebral artery, 14-3-3 COM-like IR was intense in infiltrating cells (Fig. 1A, arrow) in the thickened tunica intima and faint in the morphologically normal tunica media (Fig. 1A, asterisk), similar to the control subjects. In the carotid artery lesions of CEA specimens, 14-3-3 COM-like IR was intense in multinucleated giant cells (Fig. 1B, arrow) located mainly in the thin fibrous cap plaque, many foam cells located in the periphery of lipid-rich necrotic cores, and various cells with diverse morphologies mostly

Table 1
Function of 14-3-3 protein isoforms.

14-3-3 protein isoform	Potential significance and specific localization for atherosclerotic plaque pathology	Specificity and functional significance
β: beta	Unknown	Interacts with Sirt2 and induces the down-regulation of p53 transcriptional activity
γ: gamma	Expression: injured arteries and their associated vascular smooth muscle cells	mRNA expression was up-regulated 1–3 days after balloon angioplasty of a rat carotid artery.
ε: epsilon	Associated with vascular endothelial cell survival	Up-regulated by PPAR δ Expression: stratum lucidum in the hippocampus The deletion of gene coding is associated with Miller-Dieker syndrome.
ζ: zeta	Associated with the granule protein of human platelet dense granules Expression: small vessel walls during the development of rat brains	Exp: NFT in AD Expression: oligodendrocyte nuclei in the cerebral white matter with ischemia
η: eta	Unknown	Regulates parkin ubiquitin ligase
τ: tau	Unknown	Neuroprotective effects in models of PD
σ: sigma	Unknown	Assists the cell cycle arrest in the G2 phase Associated with tumor growth

AD: Alzheimer disease, NFT: Neurofibrillary tangles, PD: Parkinson disease.