Author's response to reviews

Title: Activation of Calpain-1 in Human Carotid Artery Atherosclerotic Lesions

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Author's response to reviews: see over
Dear Editor,

Enclosed please find the revised version of our manuscript entitled “Activation of Calpain-1 in Human Carotid Artery Atherosclerotic Lesions”, which has now been edited according to the suggestions of the two reviewers.

In the revised version we have included pictures of double staining immunohistochemistry, showing that calpain activity is colocalized with cell death in carotid artery atherosclerotic plaques. A detailed response to each point of criticism is attached below. We sincerely hope that the current improvements will make the manuscript acceptable for publication in *BMC Cardiovascular Disorders*.

**RESPONSE TO REVIEWER 1**

Major revisions

1. We agree that the tissue is heterogeneous along its length and that it would be interesting to compare multiple segments along the specimen. However, taking into account the heterogeneity of the tissue we chose to homogenize the whole plaque tissue to obtain enough material from the whole plaque for all of the experiments. The only fragment removed was a 1mm-thick slice used for histology from the most stenotic region of the plaque. As we used the whole homogenate to try to make it as representative as possible of the whole plaque, we have no more material left to describe the different regions. However, we have improved the description of the samples from symptomatic and asymptomatic patients concerning cellular composition, calcification/fibrosis, and extracellular matrix composition that we found in the studied, most stenotic, segment. This has been added on page 4-5 in the Methods section.

2. The TUNEL method has been widely used for demonstrating apoptotic cell death in different tissues. Recent publications include determination of apoptosis in myocytes (Odahima *et al.*, Circ. Res. 2007;100:1344-1352), and characterization of apoptosis in kidney epithelial HK-2 cells (Malhotra *et al.*, BMC Cardiovasc. Disord. 2008;8:9 doi:10.1186/1471-2261-8-9). Furthermore, in a previous study
we have shown that oxidized LDL induces calpain-dependent apoptosis in cultured endothelial cells, where caspase-3 was inactive but chromatin was fragmented in a fashion typical of apoptosis (ref. 12 in the current manuscript). On the reviewer’s request we attempted to perform immunohistochemical staining of plaque sections for active caspase-3, but due to technical difficulties we are currently unable to present any conclusive data on the possible activation of caspase-3 in the plaques.

3. The tissue chosen for Western blotting was not chosen in particular. The homogenates consisted of the whole plaque, except for a 1mm-thick slice used for histology from the most stenotic region of the plaque. As it corresponds to 89% of the total weight of the plaque we considered it representative of the whole plaque. We agree that it does not match up with the 11% of weight of plaque that we sectioned for IHC. Those sections can only represent what they are, and that is the most stenotic area of the plaque. According to JM Seeger et al. (J Surg Res 1995, 58:330-336), the most stenotic portion of the plaque is that which contains more cholesterol, more calcium, and less collagen. We agree that it would be interesting to study less advanced lesions. However, those lesions correspond to small stenoses that according to the current clinical practice are not operated, and therefore not removed from the patient or possible to study (based in ACAS; Endarterectomy for asymptomatic carotid artery stenosis. Executive Committee for the Asymptomatic Carotid Atherosclerosis Study. JAMA 1995;273:1421-1428 and ECST; MRC European Carotid Surgery Trial: interim results for symptomatic patients with severe (70-99%) or with mild (0-29%) carotid stenosis. European Carotid Surgery Trialists’ Collaborative Group. Lancet 1991;337:1235-1243).

4. The immunohistochemistry pictures have now been improved (new figure 2). We have performed double staining with TUNEL and anti-proteolyzed-fodrin antibody, and the pictures show that the TUNEL stain is indeed colocalized with calpain activity (Fig. 2).

Minor revisions:

1. HMEC stands for human microvascular endothelial cells, which has been stated on p. 3 in the manuscript.

2. The histological characteristics of the two groups of plaques (symptomatic and asymptomatic) are now described on p. 4-5. A second reference has been added [18] for the published data on plaque histology.
RESPONSE TO REVIEWER 2

Major issues
1. Our conclusion regarding calpain activation is based on both western blots and immunohistochemistry. The IHC staining of frozen plaque sections minimizes the risk of artifacts compared to the processing of samples for western blot, and the results from both methods still show that calpain was activated in the plaques (as evidenced by proteolysis of cytoskeletal alpha-fodrin).

2. The double-staining has been performed and is shown in Fig 2.

3. In figure 1A, the 150 kDa fragment for sample 31 is indeed weak, but the tubulin band shows that this lane contained less protein than the others. In Fig 1B, however, a double band of activated calpain is clearly visible for sample 31.

4. The blot shown in Fig. 1 includes both symptomatic (#29, 29A, 32, and 35) and asymptomatic (4A, 30, 31, 33, and 34) plaque samples. As can be observed in the blot, symptomatic and asymptomatic plaque samples appeared to contain similar bands (78 kDa) of autolyzed calpain.

5. The quality of figure 2 has been improved.

Minor issues
1. Primary antibodies used in immunoblotting have now been described in more detail in the Methods section, both under “Materials” and “Immunoblotting and calpain activity”.

2. We have now added “OD-fodrin/OD-tubulin” to the legend of Figure 3.

3. The sentence “Values are presented as mean+SD” has been deleted since it is not relevant to this manuscript. The explanation of the values shown in the box plot has been added into the figure legend of Fig 3.

Sincerely,

Isabella Pörn-Ares

PhD