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Differential expression of TRAIL and its receptors relative to calcification in AAA

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Abstract

Abdominal aortic aneurysm (AAA) is commonly associated with atherosclerosis. Human AAA tissue displays cells undergoing all stages of apoptosis. Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induces apoptosis in tumour cells but not in normal cells. It has death receptors and decoy receptors. An inhibitor of TRAIL, osteoprotegerin (OPG), is involved in osteogenesis and vascular calcification. We investigated TRAIL and its receptors in AAA compared within normal aorta (NA).

Both qualitative and quantitative analyses of calcification in AAA walls were determined using Von Kossa staining and pre-operation computer tomography (CT) scans. There was a significant difference in calcification level at different locations in the AAA wall ($p < 0.05$). Apoptosis was confirmed in AAA by TUNEL assay. A significant difference in TRAIL and its receptor expression was observed between normal aortae and AAA ($p < 0.05$). Significant differences were also observed between tissues displaying different extents of calcification for TRAIL mRNA ($p < 0.05$) by RT-PCR examination and OPG protein ($p < 0.01$) by protein blotting examination.

We propose that this pattern of expression of TRAIL and its receptors may contribute to AAA formation and calcification in the AAA wall.

Keywords: Vascular calcification; Apoptosis; Abdominal aortic aneurysm; TNF-related apoptosis-inducing ligand (TRAIL); TRAIL receptors; Osteoprotegerin

Abdominal aortic aneurysm (AAA) is the dilation of part of the aortic wall, the major blood vessel of the body. In England and Wales, death following AAA rupture accounts for 1.36% of deaths in men and 0.45% of deaths in women over the age of 65. The overall mortality rate of ruptured AAAs is about 80% [1,2]. Medial layer smooth muscle cells (SMCs) have been observed to display characteristic apoptotic, morphological and biological changes at the ultrastructural level in addition to DNA fragmentation being detected by agarose gel electrophoresis [3]. The presence of AAA is positively correlated with atherosclerosis [4] and a calcified plaque is a common feature [5]. It has been suggested that there may be a correlation between bone formation and vascular calcification, recent evidence implying that its regulation is by an active process involving certain bone related proteins, and thus showing similarities to osteogenesis [6].

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) was discovered in 1995 [7] and immediately attracted much research interest because of its capacity to induce apoptosis in tumour cells but not in normal cells. TRAIL has 5 receptors: TRAIL-R1 and R2 are death receptors (DR) [8]; and the remaining 3, TRAIL-R3, R4, and osteoprotegerin (OPG) are decoy receptors (DcR) [9]. The ability of TRAIL decoy receptors to inhibit apoptosis induced by TRAIL implies a route for potentially important clinical intervention in vivo. Bone cells and vascular cells express OPG [10,11]. OPG inhibits the recruitment, proliferation, and activation of osteoclasts,

Abbreviations: TRAIL, TNF-related apoptosis-inducing ligand; AAA, abdominal aortic aneurysms.

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the cells responsible for bone resorption, and it acts as a soluble factor in the regulation of bone mass [12]. OPG is also a decoy receptor for TRAIL, thereby acting as an inhibitor of apoptosis.

The associations between bone formation, vascular calcification, apoptosis and aneurysm formation prompted this study to investigate TRAIL and its receptors and their involvement in AAA formation.

Material and methods

Samples and reagents. With local ethics approval and patient consent, normal aorta (NA) samples were obtained from pulseless kidney donors for mRNA ($n = 8$) and protein ($n = 11$) analysis. AAA tissue for mRNA ($n = 33$) and protein ($n = 42$) analysis was obtained from AAA open repair. Samples were dissected from the outer and inner parts of the aneurysm body (enlarged aorta) and were also obtained from the proximal and distal margins of the aneurysm body to represent different stages of aneurysm development.

The AAA study group included 5 females and 26 males. Ages ranged from 59 to 96 years (mean age 76 ± 7 years). The age pattern of the cohort used closely correlated with the pattern of all AAA patients who received surgical treatments at our hospital between 1990 and 2001. Overall the occurrence of aneurysm risk factors was 55% for hypertension, 17% for hypercholesterolemia, 78% were current or ex-smokers. No patients had diabetes mellitus, hypocalcaemia or chronic renal failure. Most patients (78%) exhibited other vascular disease. This pattern is fairly typical of literature reports. The diameters of AAA were from 5 to 8.3 cm (mean diameter 6.5 ± 1 cm).

Von Kossa staining and terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) assay. Normal aorta and AAA tissue were 10% formalin-fixed and paraffin wax-embedded. Sections were cut at 5 μm and de-paraffinised then rehydrated in preparation for staining. The Von Kossa silver nitrate stain was used to detect calcium deposits in the tissue. The TUNEL method is designed to detect apoptotic bodies and was carried out according to the kit manufacturer's (Chemicon, Europe Ltd., UK) specifications. Stained sections were mounted in aqueous mounting medium for fluorescence with 4′,6-diamidinophenylindole (DAPI; Vector Laboratories Ltd., Orton Southgate, Peterborough, UK) and viewed using fluorescence microscopy. Ten contiguous fields per slide were chosen randomly and the number of apoptotic cells manually counted because the background colour of the tissues made automated counting problematic. Apoptotic index (AI) was determined.

AI = (number of TUNEL-positive cell nuclei/total number of cell nuclei) × 100%.

Protein determination in aortic wall tissue. Samples were divided into proximal, body and distal regions of AAA. Protein concentration was determined by the Bio-Rad assay. Detection of TRAIL (Chemicon, Europe Ltd., UK). TRAIL-R1 (BD PharMingen, San Diego, USA) and OPG (BD PharMingen, San Diego, USA) protein expression was carried out by Western-blot firstly. Dot blots were performed only on protein samples that contained specific protein expression bands identified by Western blot.

RT-PCR. Total RNA was isolated from 33 human vascular tissues using a total RNA isolation reagent kit (Abgene Inc., Rochester, NY). RT-PCR was performed using a commercial kit (Promega UK Ltd., Delta House, Chilworth Research Centre, Southampton) and both kits were used in accordance with manufacturers' instructions. Primers for TRAIL and its receptors were made according to published sequences.

Primer sequences for TRAIL were forward 5′-CACATTGTC TTCTCCAAACTC-3′ and reverse 5′-GTCCATGTCTATCAAGT GCTC-3′, for TRAIL-R1 forward 5′-ACTCGCTGTCCACTTTCGTCT CTGA-3′ and reverse 5′-AGGCATCCCCTGGGCCTGCTGTA-3′, for TRAIL-R2 forward 5′-GGGAGCCGCTCATGAGGAAGTT-3′ and reverse 5′-CTGGGTGATGTTGGATGGGAGAGT-3′, for TRAIL-R3 forward 5′-GAAGAATTTGGTGCCAATGCCACT-3′ and reverse 5′-C TCTTGGACTTGGCTGGGAGATGT-3′, and for TRAIL-R4 forward 5′-CAACTGGTGGGCTCCGAAAAG-3′ and reverse 5′-ACCGCATGT GGCCTAAAACGAC-3′.

Cycle conditions for TRAIL, TRAIL-R1 and TRAIL-R2 cDNA amplification consisted of 35 cycles consisting of 45 s at 95 °C, 45 s at 55 °C, and 60 s at 72 °C. The reaction for TRAIL-R3 cDNA amplification consisted of 35 cycles consisting of 45 s at 95 °C, 45 s at 58 °C, and 45 s at 72 °C. The reaction for TRAIL-R4 cDNA amplification consisted of 35 cycles consisting of 45 s at 95 °C, 45 s at 58 °C, and 60 s at 72 °C. The reaction for OPG cDNA amplification consisted of 35 cycles consisting of 45 s at 95 °C, 45 s at 55 °C, and 45 s at 72 °C. The house-keeping gene GAPDH (35 cycles consisting of 45 s at 95 °C, 45 s at 65 °C, and 45 s at 72 °C) was used as an internal control. Band densities were analysed by scanning densitometry and measured by Scion Image software.

CT scan assessment. Of the 31 AAA specimens, 24 CT scans were available for examination; the remaining seven patients had no preoperative CT scan performed. The extent of calcium deposition in the calcified plaque was scored on a semi-quantitative scale by a blinded, independent consultant radiologist. The scoring system (AC score) ranged from 0 (no visible calcium deposits) to 3 (continuous calcification) and was based on both the density and area of calcification on the scans. Each AAA CT scan was scored at the proximal (neck area of AAA), body (enlargement of AAA) and distal (lower end of AAA) regions of the AAA.

Statistical analysis. All analyses were performed by Student's t test and one way ANOVA using GraphPad Prism Instat package (Version 3.02, GraphPad Software Inc., San Diego USA). Significant differences were considered at $p < 0.05$ levels and confidence >95%. All error bars are ± standard deviation of the mean (SD).

Results and discussions

Von Kossa staining demonstrated the presence of black lamellar deposits and black granular deposits indicating severe calcification of the tunica media in all 22 AAA samples analysed (data not shown).

Analysis of the apoptosis index data revealed a significantly higher number of cells containing apoptotic bodies in the body of the AAA than in either the proximal or distal regions (Fig. 1).

Expression of TRAIL and its receptors in the human normal aorta and AAAs

Messenger RNA was measured in human NA and AAAs by RT-PCR. Since TRAIL was expressed similarly

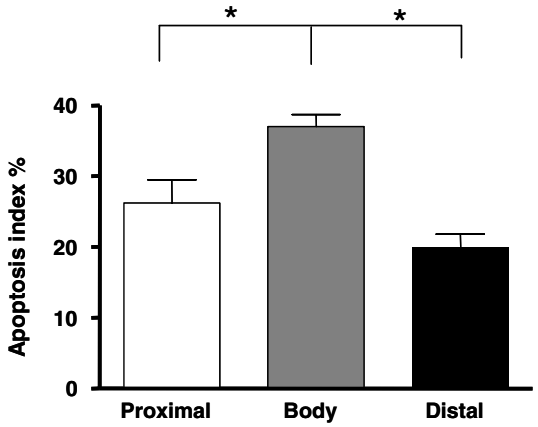

Fig. 1. Percentage of apoptotic cells (apoptosis index) by regions (proximal $n = 5$, body = 10 and distal $n = 7$). The most affected area is the body AAA ($^*p < 0.05$) and the least affected is the distal AAA.

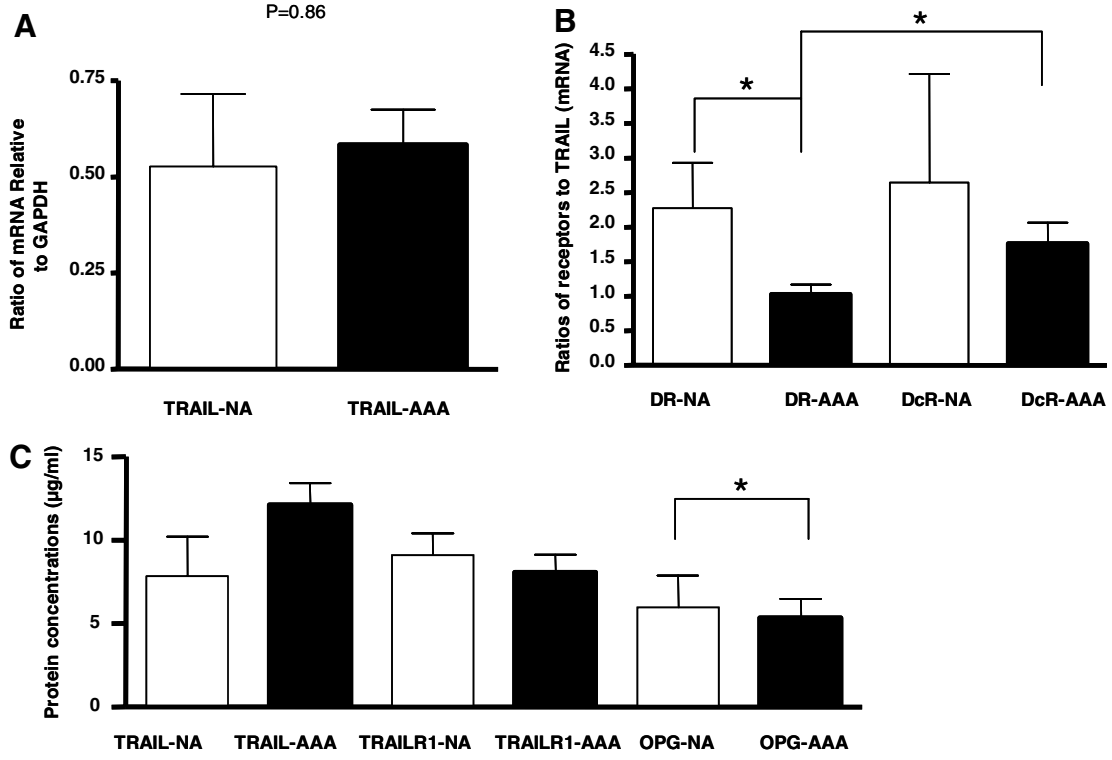

Fig. 2. Comparison of TRAIL and its receptors between normal aortae (open bars) and AAA (filled bars). (A) mRNA expression for TRAIL is expressed at similar levels in both tissues. (B) The ratios of death receptors to TRAIL and decoy receptors to TRAIL. DR-NA and DR-AAA indicate the ratios of death receptors to TRAIL in normal aorta and in AAA, respectively. DcR-NA and DcR-AAA indicate the ratios of decoy receptors to TRAIL in normal aorta and in AAA respectively. DR-AAA is significantly decreased ($^*p < 0.05$) in AAA compared to NA. DcR-AAA was expressed significantly lower than DR-AAA ($^*p < 0.05$). (C) TRAIL, TRAIL-R1, and OPG protein expression. The expression of OPG was significantly higher in normal aortae than in AAA ($^*p < 0.001$).

in human NA and AAAs (Fig. 2A) and the ratio of death receptors to TRAIL is higher in AAA than NA it follows that death receptors (DR) are higher in normal aortae (Fig. 2B). Decoy receptors (DcR) are expressed more than death receptors in AAA (Fig. 2B). We also observed the protein expression of TRAIL, TRAIL-R1 and OPG in tissue lysate, as determined by protein dot blot. OPG was significantly decreased in the human AAA tissue compared to NA (Fig. 2C).

TRAIL and its receptors expression in differentially calcified AAA samples

Calcification levels of different AAA lesions were determined by CT scan. Clearly, the proximal area is the least calcified area and the distal is the most calcified area (Fig. 3A). The expressions of TRAIL and its receptors in the three different regions of AAA were examined by RT-PCR (Fig. 3B–G).

Discussion

Although OPG expression in AAA has been reported [13], we are the first to monitor the expression of TRAIL and all its receptors in normal aorta and AAA. Furthermore we have linked this to the degree of calcification in vivo. Histological and immunohistochemical techniques confirmed the presence of both severe calcification and apoptotic bodies in the wall of AAA (data not shown). Cells were found to be undergoing apoptosis in different areas of the AAA vessel wall. However, only a very small proportion of apoptotic cells were visualised in the tissue sections. Relatively, the least apoptotic cells were found in the distal AAA (most calcified region). As the apoptotic cells are available to be observed under light microscopy for only a few minutes and apoptotic bodies can be visualised only for a few hours before they undergo phagocytosis [14], this could explain why only a small percentage of cells were found to be apoptotic. However, even the presence of a small detected percentage can still represent a considerable magnitude of cell loss [15]. The low level of apoptosis detected in the distal area may imply early apoptosis in the distal AAA before calcification occurred. This is consistent with bone formation in that osteoblasts and osteoclasts undergo apoptosis before calcium deposition occurs [16].

It was unsurprising that we detected the TRAIL system in human normal aorta and AAA considering its apparently ubiquitous expression. However, because our initial studies suggested that there were differences in expression between diseased and normal aorta we embarked on this study to investigate whether these differences could be correlated to the pathogenesis of aneurysmal disease. We con-

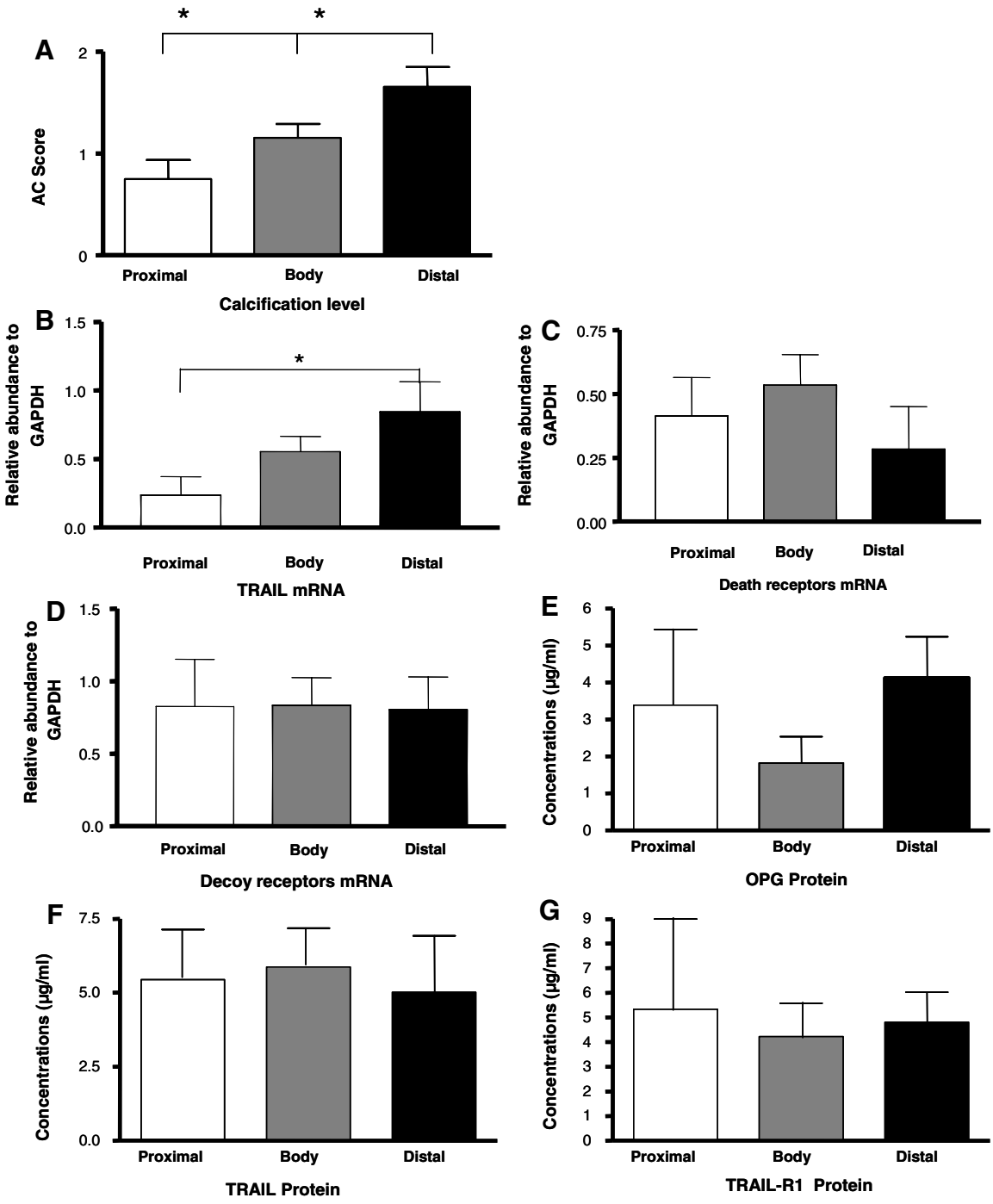

Fig. 3. (A) The AC score of proximal, body and distal lesions of AAA. Distal is the most calcified area and proximal is the least calcified area according to the CT scans. Proximal calcification level is significantly less than the distal area ($^*p < 0.05$), as is the body area ($^*p < 0.05$). (B–G) mRNA and protein expression were measured in proximal ($n = 9$), body ($n = 12$) and distal ($n = 11$) AAA samples. (B) TRAIL increased with increasing calcification level and proximal and distal areas of AAA were significantly different ($^*p < 0.05$). (C) TRAIL death receptors' mRNA expression. Interestingly, death receptors displayed the highest expression in body AAA and the lowest in distal AAA. (D) TRAIL decoy receptors' mRNA expression; these were expressed very similarly in the three AAA regions. (E) Indicates OPG protein expression. The pattern of expression was a mirror image of TRAIL death receptors mRNA. Where the TRAIL death receptors were expressed the most, OPG was expressed the least. (F) TRAIL protein was expressed very similarly in all three regions. (G) Indicates TRAIL-R1 protein expression in these regions. TRAIL-R1 was expressed mostly in the distal AAA and least in the body AAA but very similar.

sidered the study to be particularly pertinent because of the circumstantial evidence linking the TRAIL system with bone physiology and pathology, apoptosis and vascular calcification. Human vascular calcification has been shown to be a regulated process with similarities to bone modelling and remodelling [17,18]. OPG is an inhibitor of osteo-

clast formation that is found in bone cells, endothelial cells and vascular SMCs [19]. OPG-deficient mice often exhibit extensive bone fractures and medial calcification of the aorta and the renal arteries [20]. These problems are comparable with the finding of bone fractures coexisting with vascular calcification in humans [21–23]. All these factors suggest that the TRAIL system might play a role in osteoporosis and vascular calcification.

It is possible that the different calcification level of aneurysm could be a result of unsteady three-dimensional blood flow that might also stimulate calcification in AAA. A Japanese research group successfully produced a model of such three dimensional blood flow using a graphics workstation [24]. This aneurysm flow model predicts that a single asymmetric blood flow occurs in the proximal (neck) region of the AAA which coincides with the area of least calcification found in this study. In the aneurysm body, two symmetrical vortices were formed which might lead to more calcification than that found in the proximal area. In a similar way, the model proposes that as the blood passes through the wide body of the AAA into the narrower distal region, a greatly increased stress is exerted on the vessel wall possibly inducing a higher level of calcification in a similar way to how bone is strengthened by calcification when subjected to pressure and tension [25] (Fig. 4).

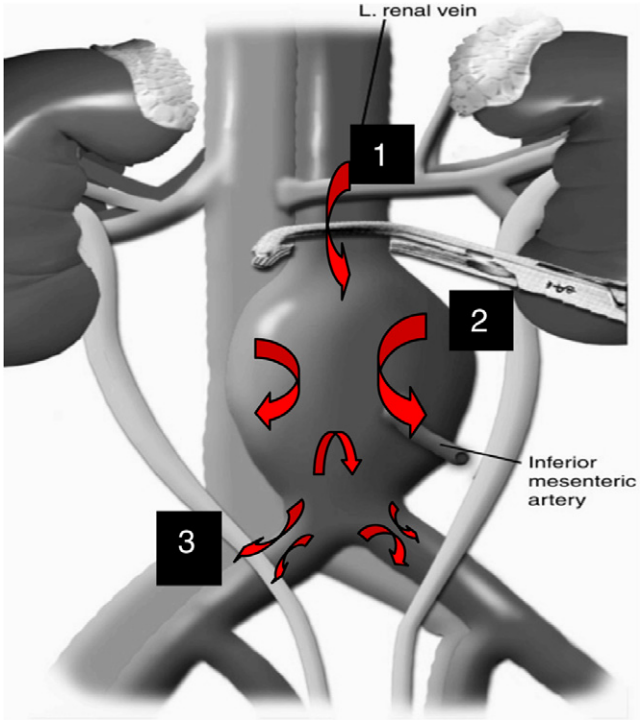

Fig. 4. Blood flows in the calcified AAA as indicated by the arrows. The No. 1 blood flow stream is through the proximal AAA and becomes No. 2 vortices in the body of the AAA. The No. 2 blood flow forms a small circulation inside the enlargement of AAA increasing the burden on the vessel wall causing more severe calcification. When No. 2 blood flow stream arrives at narrow distal area, the extra stress of the vessel wall suddenly increases No. 3. This could explain why the distal area is the most calcified region (modified from Heal, national video library).

The significant difference of mRNA and protein for the TRAIL system between normal aorta and AAA suggests that it may be involved in the initiation of abdominal aneurysm formation. The mRNA level of death receptors and decoy receptors are similar in NA. However, the significant difference between TRAIL death receptors and decoy receptors in AAAs may indicate an imbalance between these receptors with respect to the normal situation. Death receptors are decreased significantly in AAA. Excess TRAIL will therefore bind to decoy receptors which may be up-regulated in response leading to increased apoptosis.

OPG protein was significantly higher in the normal aorta compare to AAA which is not in agreement with the findings of Moran et al., who found OPG to be expressed higher in the age-matched AAA tissue ($n = 15$) than in normal aorta ($n = 5$) [13]. These differences could be due to numbers or tissue friability. We have examined more AAA samples ($n = 31$) and our normal samples for comparison were very freshly obtained from viable donor tissue rather than post mortem material as used by Moran et al. [13].

The potential morbidity of an aneurysm increases with increasing size. Larger aneurysms are more prone to rupture and cause life-threatening complications. TRAIL and its receptors show greatest expression in the larger aneurysm group (diameter greater than 69 mm, data not shown). This early data suggests a possible correlation between TRAIL and its receptors with aneurysm size that warrants further investigation.

TRAIL expression was positively correlated with calcification levels. Death receptors were expressed most strongly in the body of the aneurysm equating to the general situation of a developing aneurysm where apoptosis might be up-regulated. The lowest level of death receptors were found in the distal region that would consist of damaged apoptotic cells incapable of expressing new protein. This is consistent with previously published results of bone related activators of calcification which were restricted to advanced, calcified lesions suggesting that vascular calcification is the result of time and plaque-stage-restricted activation and continuous inhibition [26].

Correspondingly, decoy receptors: TRAIL-R3, TRAIL-R4 and OPG were expressed consistently in all regions of AAA walls. This accords with bone related inhibitors of calcification which were found to be exhibited at all stages of human atherosclerosis [26]. OPG protein was up-regulated in the less calcified areas and down-regulated in severely calcified areas. This feature might be representative of a counter-regulatory mechanism of OPG limiting apoptosis in the less calcified areas and therefore the initiation of calcification. Since OPG has been found to play a role in the process of vascular calcification in an animal model [10], it may be that OPG also plays a role in human AAA pathogenesis too. The limitation of this study is that samples received were from the late stage of AAA formation due to the criterion for surgical intervention being limited to a certain minimum size. Ideally testing samples from

small aneurysms (<5 cm) would help to further evaluate the role of TRAIL and its receptors in the development and formation of AAA.

Nevertheless, combining the findings of highest calcification in the distal AAA, the theory of the blood flow dynamics and the apoptotic index of the different areas, the following possibilities can be suggested. Apoptosis that occurs in the distal area (where aneurysm occurred firstly, early stage) might be involved in the arterial wall remodelling. Blood flow dynamics increases aortic wall stress causing injury of the arterial wall leading to atherosclerosis. In addition, the histological features of thinning of the *tunica media* and the disappearance in the elastic lamina are contributory to aneurysmal changes. After formation of an aneurysm apoptosis continues to occur frequently in the neck of AAA (proximal area), which might explain why more apoptosis was found in the proximal than in the distal area in the AAA samples examined.

Conclusion

These data indicate that TRAIL could be a primary signalling molecule or involved in the pathogenesis of AAA enlargement. The data also suggest that inhibition of TRAIL may be a therapeutic strategy to prevent destruction of tissue by TRAIL death receptors in AAA. OPG is in use in a clinical trial on bone metabolism without adverse effect [27]. Therefore its use as a therapeutic agent in the prevention of AAA enlargement could be considered to study the in vivo effects of the down-regulation of apoptosis induced by TRAIL receptors.

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