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Quantitative analysis of the expression of caspase 3 and caspase 9 in different types of atherosclerotic lesions in the human aorta

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ABSTRACT

The existing data on apoptotic processes in human atherosclerotic lesions is insufficient and is often contradictory. This study was undertaken to evaluate the levels of the expression of key apoptosis-related genes, namely, caspase 3 (CASP3) and caspase 9 (CASP9) in the normal (non-atherosclerotic) intima of the human aorta in comparison with those in different types of atherosclerotic lesions. Twenty-five autopsy samples of thoracic aorta were examined by polymerase chain reaction (PCR) analysis. The study revealed that the expressions of CASP3 and CASP9 genes were changed in different types of atherosclerotic lesions in course of the progression of the disease, but not in a unanimous way. The mRNA expression of CASP3 was found to be steadily decreasing with the progression of atherosclerosis while the expression of CASP9 showed a pattern which can be described as a "bell-shaped" relationship between gene mRNA expression and the type of atherosclerotic lesion, with the maximum being observed in fatty streaks. The fall in CASP3 expression may be associated with cellular senescence as well as with the domination of necrotic processes in atherosclerotic lesions, as shown by electron microscopic analysis. Our study provides novel quantitative data on the expression of CASP3 and CASP9 genes in different atherosclerotic lesions in the human aorta and thus, might assist in better understanding of the processes occurring during the development of lesions in human atherogenesis.

1. Introduction

Atherosclerosis is a complex disease which can be described as an excessive fibro-fatty, proliferative, inflammatory response to damage of the artery wall, involving several cell types, including smooth muscle cells, monocyte-derived macrophages, dendritic cells and lymphocytes (Ross, 1999; Schwartz et al., 1993; Vanderlaan and Reardon, 2005). Atherosclerosis is also thought to be a disease of aging reflecting premature systemic biological aging as well as cellular senescence occurring in the arterial wall (Wang and Bennett, 2012). Examinations of atherosclerotic plaques provided evidence of reduced cell proliferation, irreversible growth arrest and apoptosis, elevated DNA damage, etc. (Vanderlaan and Reardon, 2005; Wang and Bennett, 2012).

There is growing evidence that cellular senescence might promote atherosclerosis (Wang and Bennett, 2012). It has been suggested that the expression of apoptosis-related genes in arterial wall tissue may be one of useful indicators for the evaluation of molecular characteristics of cellular senescence during atherogenesis (Wang and Bennett, 2012), however, the data on apoptotic processes in human atherosclerotic lesions is notably insufficient and is often contradictory (Andrés et al., 2012; Bitto et al., 2010; Clarke et al., 2007; Kavurma et al., 2008; Konstadoulakis et al., 1998; Martinet et al., 2002; Matheeussen et al., 2013; Rowe et al., 2000; Sakakura et al., 2013).

In this study using polymerase chain reaction (PCR) technique we analyzed apoptosis by means of the evaluation of levels of the expression of key apoptosis-related genes, namely, CASP3 and CASP9 in the normal (unaffected by atherosclerosis) intima of the human aorta in comparison with those in different types of atherosclerotic lesions. Sequential activation of caspases plays a central role in the execution of cell apoptosis, during this event CASP3 interacts with CASP9 (Johnson and Jarvis, 2004; Konopleva et al., 1999; Kuida, 2000; Porter and Jänicke, 1999; Yuan et al., 2011).

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2. Materials and methods

2.1. Tissue specimens

Twenty-five thoracic aorta samples were collected in a period between 1.5 and 3 h after sudden death at the autopsy from 19 males and 6 females aged between 53 and 72 years (mean age, 66.3, SD = 6.2). The study was carried out in accordance with the principles outlined in the Helsinki Declaration of 1975, as revised in 1983. The study protocol was approved by the Ethics committee of the Institute for Atherosclerosis Research, Moscow.

The aortas were opened longitudinally and washed with phosphate-buffered saline (PBS), pH 7.6. The unaffected regions of the aortas and those with atherosclerotic lesions were identified macroscopically according to the classification of the Atherosclerosis Council of the American Heart Association (Stary et al., 1994, 1995), utilizing the corresponding histological evaluations as used previously (Bobryshev et al., 2011; Orekhov et al., 2010). Areas, unaffected by atherosclerosis, were defined as tissue samples with smooth luminal surfaces. Zones with initial atherosclerotic changes (type I lesions) corresponded to the luminal parts of aortas with a smooth yellowish surface with occasional small yellow spots. Small aggregates of extracellular lipid droplets were present in the connective tissue matrix, as was confirmed by following routine histological examination. According to histology, apart from resident cells, the initial lesion foci were characterized by an increased number of mononuclear cells, in contrast to the visually intact intima. Fatty streaks (type II lesions) were defined as yellow strips and spots that slightly protruded over the vessel surface, often merging into larger structures and forming lesion clusters. In tissue sections, the presence of lipids was identified inside both round-shaped macrophage-like cells and spindle-shaped smooth muscle cells. Lipofibrous plaques (type Va lesions) were defined as spherical or elliptic protrusions of yellowish or nacreous color. Microscopically, they included accumulated intracellular lipids and increased amounts of the extracellular matrix. Lipofibrous plaques contained a bulky necrotic core covered by a connective tissue layer and also included zones that morphologically resembled fatty streaks. Fibrous plaques (type Vc lesions) were defined as considerably protruding, rounded or oval, and pearl-colored formations. They were mostly composed of a crude connective tissue matrix with embedded cells, and the lipid component was rare. Thus, the type of lesions was identified according to commonly accepted classification (Stary et al., 1994, 1995).

2.2. Polymerase chain reaction analysis

Following the macroscopic identification, aortic tissue samples (unaffected tissues, initial lesions, fatty streaks, lipofibrous plaques, and fibrous plaques) were taken, aortic intima was separated from medial layer mechanically, and total RNA was extracted from intimal samples with TRIzol® reagent (Invitrogen, USA), using the methodology described elsewhere (Hummon et al., 2007). The concentration and purity of extracted RNA was determined using the NanoPhotometer® (Implen). The RNA extracts were of high purity, free of DNA contamination, and allowed highly sensitive and specific detection of mRNA by a reverse transcription-PCR. The synthesis of cDNA was performed using ImProm-II™ Reverse Transcription System kit (Promega, USA).

The levels of CASP3 and CASP9 gene mRNA expressions were estimated by quantitative real-time PCR amplification of reverse-transcribed mRNA fragments. Quantitative real-time polymerase chain reaction (PCR) was carried out in triplicates for each gene using the reaction mixture for RT-PCR with SYBR Green I (Syntol, Russia) on 7500 Fast Real-Time PCR System (Applied Biosystems, USA). Housekeeping genes GAPDH and CAP1 were used to assess the quality of the synthesized cDNA and to normalize PCR results. The primer sets used for GAPDH were 5′-actttggtatcgtggaaggact-3′and 5′-gtagaggcagggatgatgttct-3′, for CAP1 5′-attccctggattgtgaaatagtc-3′ and 5′-attaaagtcaccgccttctgtag-3′. The primer sets for CASP3 were 5′-gtggaattgatgcgtgatgtttc-3′and 5′-gtccagttctgtaccacggc-3′, while the primer sets for CASP9 were 5′-cacccagaccagtggacatt-3′and 5′-tgctcaggatgtaagccaaatct-3′.

Statistical analysis was performed using SPSS v. 14 (SPSS Inc., USA). The methods of one-way analysis of variance, cross-tabulation, and correlation analysis were used. The comparisons of mean values were performed using Mann–Whitney U-test and Wilcoxon's statistics. The significance of differences was defined at a 0.05 confidence level.

2.3. Electron microscopy

Electron microscopy was used to examine the structural appearance of cells in different types of atherosclerotic lesions. For further ultrastructural analysis, several small samples of intimal tissue (approximately 1 mm^3, each) from different types of atherosclerotic lesions were dissected, were first fixed in 2% glutaraldehyde in PBS, then post-fixed in 1% OsO_4, and eventually were routinely embedded into Araldite blocks, as used previously (Bobryshev et al., 2011; Orekhov et al., 2010). Ultrathin sections were stained with uranyl acetate and citrate lead, and were examined using a Hitachi H7000 electron microscope.

3. Results

In total, 78 intimal samples taken from 25 aortas were analyzed; among those there were 18 samples of normal (unaffected by atherosclerosis) intima, 20 initial lesions, 16 fatty streaks, 16 lipofibrous plaques, and 8 fibrous plaques. Five aortas were characterized by the presence of all types of atherosclerotic lesions along with the presence of unaffected regions. Furthermore, 9 aortas had unaffected regions and nearly all types of atherosclerotic lesions, except the presence of lipofibrous or fibrous plaques. Seven aortas did not have unaffected regions but contained combinations of different types of atherosclerotic lesions. The remaining 4 aortas had only one type of atherosclerotic lesion (initial lesion or fatty streak), along with unaffected areas.

The results of the measurement of mRNA expression of CASP3 and CASP9 genes in areas, unaffected by atherosclerosis, and in atherosclerotic regions of the human aortic intima are summarized in Fig. 1. As it is seen in Fig. 1, the level of CASP3 gene expression demonstrated a monotonous decay during the progression of atherosclerotic lesions; with the highest level being observed in unaffected intimal tissue and with the lowest one observed in fibrous plaques ($p = 0.0018$ for the trend). The last differed significantly from areas of intima unaffected by atherosclerosis ($p = 0.041$) and from initial lesions ($p = 0.019$), but difference did not reach statistical significance when compared to fatty streak ($p = 0.098$) and lipofibrous plaque ($p = 0.061$). There were no statistically significant differences in CASP3 mRNA expression between other types of atherosclerotic lesions in paired comparisons (Fig. 1A). There were significant correlations in CASP3 gene expression in unaffected intima and initial lesions ($r = 0.643$, $p = 0.018$) and in fatty streaks and lipofibrous plaques ($r = 0.593$, $p = 0.033$), accordingly.

The levels of CASP9 gene expression were found to be quite similar in all types of atherosclerotic lesions and in unaffected intima, except a different level of the expression for fatty streaks, where nearly 2-fold increase was observed ($p = 0.084$, $p = 0.030$, $p = 0.002$, and $p = 0.046$ vs unaffected intima, initial lesions, lipofibrous plaques, and fibrous plaques, respectively) (Fig. 1B). There was significant correlation in CASP9 gene expression in fatty streaks and lipofibrous plaques ($r = 0.933$, $p < 0.031$).

Electron microscopic analysis of the normal intima demonstrated that there were very few cells showing signs of destructive changes while the examination of ultrathin sections of all types of atherosclerotic lesions demonstrated that cells showing signs of destructive alterations were frequently present, with most dead (completely destroyed) cells seen in zones of lipid/necrotic cores in atherosclerotic plaques. Notably, no cells, the structure of which would be indicative of that that these