

Original

Modified Quantitative Detection of Apoptosis in Coronary Atherosclerosis

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Key Words

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Background. Apoptosis, or programmed cell death, is a physiological cell death process that allows a tissue or organ to remove unwanted cells and respond to the maintenance of stable cell numbers in tissues even in primary atherosclerotic or restenotic lesion of coronary artery. Previous studies on the apoptosis of coronary atherosclerosis are usually prone to run into qualitative description without the aid of "quantitative" evaluation. To eliminate such subjective errors, we have designed a triple immunofluorescent stain as assessed by digital camera and computer-aided analysis system so that the actual quantitative apoptotic expression of coronary atherosclerotic development can be more precisely evaluated.

Methods. Specimens of coronary artery were obtained from consecutive patients undergoing coronary endarterectomy or cardiac transplantation. Twenty-seven blocks of paraffin tissue specimens from 16 patients were analyzed. According to the American Heart Association (AHA) classification of atherosclerotic lesion, type I, II, III lesions were defined as early lesions and the other three types: IV, V, and VI as advanced lesions. Apoptosis and cell types were recognized simultaneously by triple immunofluorescent stain combined with Hoechst, Terminal deoxynucleotidyl transferase-mediated dUTP Nick End-Labeling (TUNEL) and smooth muscle cell (SMC) marker staining. Apoptotic index deduced from TUNEL-positive nucleus number divided by Hoechst-positive nucleus number. Apoptotic cell-type index deduced from TUNEL-positive nuclei, which are surrounded by cell type fluorescent marker, divided by total TUNEL-positive nuclei.

Results. Fourteen early lesions and 13 advanced lesions of tissue specimens were analyzed in this study. The mean apoptotic index of early lesions was $2.60 \pm 1.72\%$, which is significantly lower than that of the advanced lesions ($7.42 \pm 3.07\%$). The apoptosis between the tapering portion and lesion portion obtained from 10 coronary endarterectomy specimens was also significantly different ($2.59 \pm 1.90\%$ vs $8.10 \pm 3.20\%$). In either early or advanced lesions, the predominant cell type of apoptosis was SMC.

Conclusions. The identical counting and quantification analytical method we designed is more accurate and quantitative than the traditional investigation in detecting and affirming the "homeostasis" role of apoptosis in the atherosclerotic pathogenesis process of coronary artery disease.

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Apoptosis of vascular smooth muscle cells (SMCs) has been identified as an important process in a variety of human vascular disease including atherosclerosis, arterial injury, restenosis after angioplasty and dilated cardiomyopathy, and even in acute myocardial infarction (AMI) or heart failure status. Isner *et al*¹ and Han *et al*² have confirmed that apoptosis did modulate the cellularity of lesions that produce human vascular obstruction, particularly those with evidence of more extensive proliferative activity. The recognition of apoptosis in normal or rapidly proliferating cell populations suggested its potential role in the maintenance of stable cell numbers in tissues with various degrees of proliferative activity. To maintain normal wall shear stress, the vessel is remodeling even from the early stage of fatty streak or atherosclerosis-prone lesion to advanced atheroma. During this process, the balance between cell replication and apoptosis should attain a "homeostasis" status.

The significance of apoptosis has mostly been studied by using the Terminal deoxynucleotidyl transferase-mediated dUTP Nick End-Labeling (TUNEL) assay that detects DNA strand breaks in tissue sections and allows quantification of apoptotic cells by light microscopy. However, there are regrets with traditional photographic counting and quantification of apoptotic index. Due to the limitations of personal and technical factors, the apoptotic expression announced in a more "qualitative" than "quantitative" way. The reported percentage of TUNEL-positive nuclei of human coronary atherosclerosis varies a lot, ranging from < 2%^{1,3,4} up to 30%,^{2,5,6} it seems to be that the TUNEL assay is prone to false positive or negative findings.

To eliminate the quantitative variation and to obtain reliable and reproducible results in quantification of apoptosis, the specificity of apoptosis can be substantiated by combining the modified triple immunofluorescent staining with digital camera and computer-aided analysis system. Therefore, the apoptotic expression of coronary atherosclerosis can be described in a more scientific and objective way.

The present study was elaborated to investigate whether the variations of apoptotic nuclear index were

significantly associated with different stages of human coronary atherosclerosis, furthermore, the more advanced atherosclerosis area needed more "apoptotic process" to maintain the cellularity balance.

Methods

Specimens of coronary artery were obtained from 16 consecutive patients (5 females, 11 males, mean age 57.4 years, in a range from 6 to 75 years) undergoing coronary endarterectomy (n = 10) or cardiac transplantation (2 donors and 4 recipients). Twenty-seven blocks of paraffin-embedded tissue section were analyzed.

Tissue preparation and histological analysis

Specimens of coronary artery were dissected gently to free adhering tissues, rinsed with ice-cold phosphate buffered saline (PBS), immediately fixed in 4% buffered paraformaldehyde overnight at 4 °C, and subsequently dehydrated in sequential 50%, 70%, 80%, 90% and absolute alcohol washes. After embedding in paraffin, the samples were cut into 3-5 μm serial sections, and fixed on aminoalkylsilane (AAS)-coated slide.⁷

The microscopic evaluation was performed by two observers who were blind to the clinical data. Specimens were evaluated for the presence of intimal hyperplasia, fibrous plaque, thrombus, plaque hemorrhage, foam cells, calcium, SMC, media, and adventitia. Intimal hyperplasia was defined as the presence of abundant SMCs in a proteoglycan matrix.

Hematoxylin and eosin staining for classification of coronary atherosclerosis

Following staining with hematoxylin and eosin reagent, histological sections were observed and photographed under the light microscope. Thereupon staging of human atherosclerotic lesions was based on their histological composition and structure and perceived as characteristic gradations or stages from initial minimal changes to lesions associated with clinical

cal manifestation. According to the classification of American Heart Association (AHA), the atherosclerotic lesions are categorized into 6 different types.⁸ Type I, II, III lesions are defined as early lesions and the other three types, IV, V, and VI, are defined as advanced lesions.

DNA gel electrophoresis

Tissue specimens were homogenized in liquid nitrogen and lysed in genomic DNA isolation reagent (GibcoBRL, Life Technologies, Green Island New York, U.S.A). Then total DNA was precipitated by absolute ethanol. After centrifugation, the DNA pellets were dissolved in 8 mM NaOH solution and electrophoresed in 2% agarose preimpregnated with ethidium bromide. Gel was visualized and photographed under transmitted ultraviolet (UV) light with a Polaroid camera.

Transmission electron microscopy

Examination of transmission electron micrographs confirmed and extended the observation made by light microscopy. Following fixation, each speci-

men was washed thoroughly in 0.1 M phosphate buffer and postfixed in 0.1% buffered OsO₄ for 2 hours at 4 °C. The samples were dehydrated through a graded ethanol series followed by propylene oxide. Each sample was embedded in Epon 812. Ultrathin sections were placed on formvar grids, stained with uranyl acetate and lead citrate and viewed with JEOL JEM-2000EXII transmission electron microscope.

Triple immunofluorescence stains for Hoechst/TUNEL/ α -SMC-actin cell marker antibody

This “tricolor” immunofluorescent staining method combined with a simultaneous analysis system reveals detailed information on a single tissue slide level.

Triple immunofluorescent staining protocol (Fig. 1)

Procedure 1

Deparaffinize and rehydrate the tissue slides with standard procedure:

Xylene three times for 5 minutes, sequential absolute alcohol, 95%, 85%, and 75% ethanol each for 3

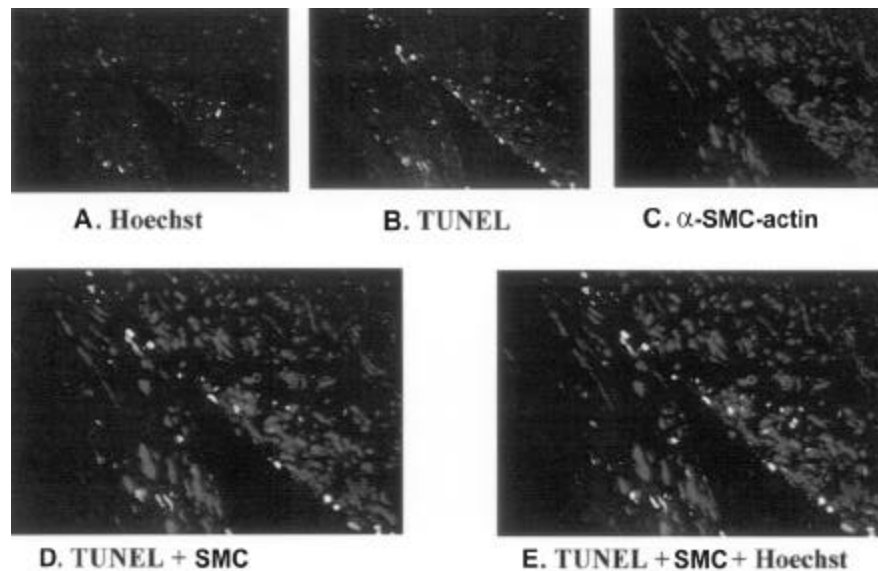


Fig. 1. Triple immunofluorescent staining for Hoechst/TUNEL/ α -SMC-actin cell marker antibody (A) Hoechst staining under the UV light exposure. (B) TUNEL technique conjugated with FITC chromogen. (C) Staining with α -SMC-actin cell marker antibody. (D) TUNEL + SMC cell type marker. (E) TUNEL + SMC + Hoechst: the final merged “tricolor” image.

minutes, and finally wash in water for 3 minutes.

Procedure 2

Pretreatment with microwave:

Place slides in a plastic jar containing 200 ml 0.01 M citrate buffer, pH 6.0 and apply 800W microwave irradiation for two minutes. After heating up to around 86 °C, cool rapidly by adding 80 ml distilled water. Immediately transfer the slides and immerse into PBS solution at room temperature (RT). Be careful that proteinase K treatment is contraindicated in this period. The whole procedure has been recommended for microwave enhancement of TUNEL labeling method by Lucassen *et al.*⁹ and Strater *et al.*¹⁰

Procedure 3

Labeling:

The non specific material is blocked with an antibody diluent (Dako Dako. Corp., Carpinteria. CA) at 37 °C for 15 minutes. Soon afterwards, rinse sample twice in PBS, add TUNEL reaction mixture (B.M.Cat. California. U.S.A. No 1684795 bottle one: bottle two = 1:9) and incubate at 37 °C for 1 hour. Again wash twice in PBS buffer, dribble monoclonal Mouse anti-human- α -SMC-Actin antibody (Zymed Cat. Sigma. Chemical CO. St. Louis. MO. No 08-0106) and incubate at 37 °C for 1 hour. Once more do two washes in PBS for 5 minutes, and cover the sections with secondary antibody solution: Goat anti-mouse IgG Perkin-ELMER Life Science Inc Massachusetts. U.S.A. (Jackson ImmunoResearch Laboratories Cat. No 115-026-062) 1:50, RT, for 1 hour. Then, wash in PBS buffer for 5 minutes twice again, stain with Hoechst 33342 Germany. Frankfurt (Sigma B-2261, 5 ug per milliliter diluted in PBS) for 15 minutes at room temperature. Finally, wash off in PBS for 5 minutes two times and mount the slide over the stage of fluorescence microscope for further evaluation.

HOECHST 33342 (nuclear DNA labeling):

A bisbenzimidazole dye binds to adenine/thymine rich regions in the minor groove of DNA and displays the nucleus as white speck under the exposure of fluorescent UV light.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling):

Apoptosis, a genetically encoded cell death program, was recognized nearly 30 years ago by Kerr¹¹ as a distinctive morphology that he called "shrinkage necrosis." Apoptosis is best characterized biochemically by the cleavage of genomic DNA into nucleosomal fragments of 180 base pairs (bp) or multiples that are readily detected as a DNA ladder by gel electrophoresis of lysate from cells grown in culture.¹² This approach is less applicable for examination of whole tissue specimens. Identification of apoptosis in tissue sections has been greatly facilitated by specific immunolabeling of nuclear DNA fragmentation with terminal deoxynucleotidyl transferase (TdT).¹³ This method, TUNEL, has been widely used in the detection of apoptosis in atherosclerosis^{2,14} and can be detected as fluorescent green dot if we use conjugated FITC as secondary antibody.

Anti- α -SMC-actin (Zemed Laboratories Inc. Sigma. Chemical CO. St. Louis. MO: Clone: IA4):

The mouse-anti-human antibody reacts with the alpha isoform of SMC actin and does not cross-react with skeletal, cardiac muscle actin, or other mesenchymal and epithelial cells. It does not react with other components of the cytoskeleton, either. In our study, we used Rhodamine-conjugated secondary antibody: (TRITC) conjugated AltiniPure F (ab) 2 Fragment Goat Anti-Mouse. IgG (H+L) (minimal cross-reaction to human, bovine and Horse Serum Proteins) to react with anti- α -SMC actin primary antibody, which will display as fluorescent red halo grain. One stenotic coronary segment was used as the internal positive control to show consistent positive staining for all sections. As a negative control, the staining procedure was performed without addition of the primary antibody. A spare normal coronary segment was also used as a negative control, showing no proliferation or apoptosis throughout the different staining sections.

Procedure 4

Recognition of apoptotic nuclei:

Apoptosis was recognized by intravital staining with Hoechst 33342 (Ho342) and by TUNEL. To con-

firm apoptotic mode of cell death, the morphology of respective cell was examined and double-checked carefully by fluorescent microscopy at x200 and x400 magnification. TUNEL technique combined with Hoechst stain for detection of apoptosis in tissue section, TUNEL and Hoechst 33342, have been used independently to identify apoptosis. Both have their own advantages and disadvantages. TUNEL specifically labels dying cells, yet a low background makes comparison between labeled-nucleus and neighboring normal cells difficult and causes disoriented tissue sections. Hoechst 33342 binds all DNA, and can, therefore stain all nuclear materials to identify apoptotic nuclei, but the analysis is laborious. The combination of both fluorescent labels allows the initial recognition of apoptotic cells by TUNEL technique to display the selective apoptotic nuclei. Soon afterward by switching to the UV filter, the TUNEL-positive nuclei can be configured distinctly with neighboring normal nuclei to access the morphology and size change. Hence Hoechst 33342 acts like a counterstain, allowing identification of nuclear anatomical structures, and quantitative comparison between TUNEL-positive and normal cells. This technique enables easier and more objective calculation.

Procedure 5

Identification of the type of apoptotic cells:

Computer-saved images from staining with specific monoclonal antibodies against α -SMC actin, or CD 68 (macrophage marker) and TUNEL were merged as one scene. The TUNEL-labeled nuclei and nuclear fragments that were enclosed by a cage of cell marker material (Rohdamin, red color) indicate smooth muscle apoptosis. Both macrophages and SMCs were found to bear the markers of apoptosis.

Procedure 6

Counting area and quantification:

Tissue sections with triple fluorescent stain are investigated by fluorescent microscopy at x200 magnification with a standard field size. The arterial intima wall is divided into 5 sectors of equal size, and center fields are captured randomly in each sector. The image is first photo synthesized with digital camera, then

recorded, analyzed and adjusted by the assist of a color-image computer system (freemax image color). No picture will be saved until the same image condition of the original microscopic view is attained. Four pictures are stored in MO disk for each counting area. The first one is taken via UV light filter---that is for Hoechst stain, the second one via FITC filter---for TUNEL stain, the third one via Rhodamin filter---for anti- α -SMC-actin stain. Finally these three pictures are mingled into a triple color film. The whole procedure is demonstrated in Fig. 1.

The apoptotic index and apoptotic cell-type index are calculated in the following rules:

Apoptotic index

Apoptotic nuclear number (TUNEL)/total DNA nuclear number (Hoechst).

Apoptotic cell-type index

Apoptotic-positive nuclear α -SMC-actin antibody labeled area/apoptotic nuclear number (TUNEL).

Statistically, we use the average of these original indexes for further calculation. The apoptotic index of this tissue section is deduced from mean of these 5 equal sectors.

Measuring and counting computer technology is applied to facilitate counting and calculation without altering the original image presentation. The operating procedure and some functional icons are introduced briefly in the following:

Nuclear counting

Since the monochrome image is more precise for counting, we usually use the Convert to Gray Scale in the Spatial Calibration command window to transform our color image into a monochrome one. Then the Contrast Enhancement window is used to adjust to the visual qualities of our image. Because the counting area is only focused in the intima layer, we should delineate the measured area first. The function of Free form AOI (area of interest) Tool will help us to separate the intima from the media area and confine the counting area in the sketched polygonal territory. Then we use Count/Size to count TUNEL-positive nucleus and/or Hoechst-positive nucleus in the se-

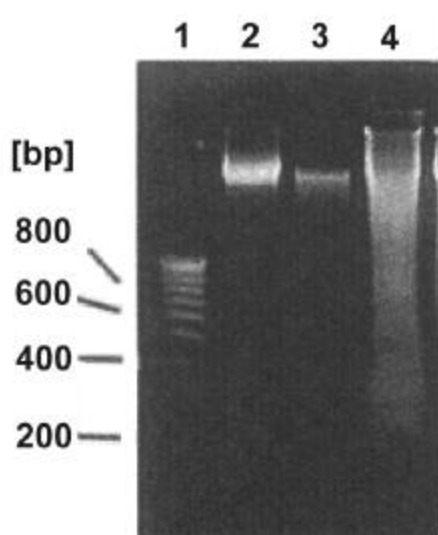


Fig. 2. Agarose gel electrophoresis analyses by DNA ladder for maturation. Lane 1: DNA marker; Lane 2: the early lesion in a patient with low cholesterol level; Lane 3: the early lesion in a patient with high cholesterol level; and Lane 4: late lesion of atherosclerosis.

lected size range.

The image merging

To identify the apoptosis nucleus or the cell types, we use the Image Operation-AND to perform a logical “and” between our images, and used the Image overlay-Merge to conglomerate two images.

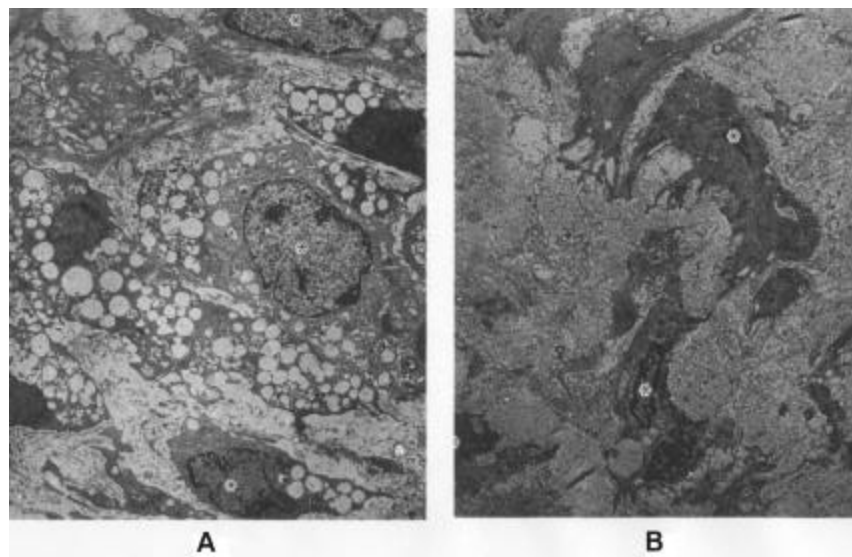


Fig. 3. Transmission electron micrographs of (A) non-apoptotic in early lesion atherosclerosis, (B) apoptotic in late lesion atherosclerosis.

Statistical analysis

Data are expressed as mean \pm SEM. Univariate analysis of mean data between early lesion and advanced lesion groups was performed by the Mann-Whitney test. Univariate analysis between tapering portion and lesion portion of specimens of coronary endarterectomy was performed by Wilcoxon signed ranks test. Differences between groups were considered significant at a probability value of less than 0.05 (two-sided).

Results

Fourteen early and thirteen advanced lesions of coronary atherosclerosis were examined. The early lesions were almost located at the mild fatty streak or atherosclerosis-prone lesions. The advanced lesions included calcification and thrombosis with moderate to severe stenosis. Apoptotic cells were found in all stages of atherosclerosis, including initial lesions, atheroma and fibrotic lesions.

Evidence of apoptosis by gel electrophoresis

After experimental periods, the tissues from early lesion and late lesion groups were collected, cellular

DNA was extracted and agarose gel electrophoresed was performed to analyze DNA fragmentation. The results are demonstrated in Fig. 2. In the early lesion group, there was no evidence of DNA fragmentation. On the other hand, apoptosis was identified in the late lesion group by DNA ladder formation.

Evidence of apoptosis by transmission electron microscopy

Under transmission electron microscopy examination, cells in the intima of the early atherosclerotic specimens did not show the characteristics of apoptotic cells. (Fig. 3A). In the late atherosclerotic specimens, many intimal cells were apoptotic, exhibiting condensation of chromatin, which are ultrastructural characteristics of apoptosis. (Fig. 3B)

Apoptosis detected by triple immunofluorescence stains for Hoechst/TUNEL/ α -SMC-actin cell marker antibody

The majority of apoptotic cells were present in the intima. However, apoptotic cells varied in number and distribution pattern with different types of tissue specimens. In the early lesions of coronary atherosclerosis, few apoptotic cells were found in the intima. The mean apoptotic index was $2.60 \pm 1.72\%$, significantly lower than in the advanced lesions ($7.42 \pm 3.07\%$, $p < 0.01$). The difference of apoptosis between the tapering portion and lesion portion obtained from coronary endarterectomy specimen was significant ($2.59 \pm 1.90\%$ vs $8.10 \pm 3.20\%$, $p < 0.01$). SMC is the predominant cell type of apoptosis in either early or advanced lesion.

Discussion

Apoptosis is a physiological cell death process that is important for normal development and involves in many pathological conditions. Atherosclerotic disease is a progressive and systemic disease, in which pathological accumulation of cells in the intima may result in migration and proliferation of smooth

muscle cells, macrophages and lymphocytes. The apoptosis in the coronary artery has proved to be located mainly in the intima layer.^{1,2,3,5} Since different stage of coronary atherosclerosis can be harvested from consecutive patients undergoing coronary endarterectomy or cardiac transplantation, our human specimens are the most suitable material for apoptosis research. Besides, coronary endarterectomy is a standard and routine procedure for diffuse atherosclerotic lesion which is not suitable for coronary artery bypass grafting. The whole hyperplastic or calcified intima and part of the thin layer of subintima tissue with atheroma plaque should be removed.

The requirement of "adequate" endarterectomy is critical for successful lumen reconstruction and the "endarterectomy" procedure could not be regarded as being "completed" until the distal tapering portion of endarterectomized atherosclerotic artery is retrieved and distal healthier intima is exploded with no more intima flap left. Comparing these two endarterectomized segments, we observed a significant difference of apoptosis exhibition between groups of tapering and severe lesion portions. Pathologically, this phenomenon could be another criteria of the "completeness" of endarterectomy procedure. Higher apoptotic index is still present in advanced lesion of transplant source, as confirmed by the earlier studies of Han *et al* and Cai *et al*.^{2,15} However, prior studies have several mistakes. Some material is from the atherectomy specimens and is oversimplified in the "snapshot" of human atherectomy tissue sampling. Being a tubular structure with eccentrically located atheroma plaque, the remodeling process of coronary topography alters the local hemodynamic pattern, which in turn influences the apoptotic manifestation in different regions. Therefore, the atherectomy specimens can't display the real apoptotic manifestation of the whole coronary atherosclerotic artery.

In this study, the apoptotic index of the tissue section was deduced from the mean value of five equal sectors. Statistically, the inferential index was closer to the real coronary apoptotic status. In addition, the traditional counting and calculation of apoptotic index is more "qualitative" than "quantitative" as mentioned above. Unfortunately, the studies of the acute re-

response to arterial injury are not possible in humans and long-term serial change is difficult to follow up. This triple stain can help us identify positive nucleus and calculate the index of apoptosis cells and the apoptotic cell types in a single slide section to achieve higher accuracy than the serial tissue section. Conjoined with three different fluorescent colors, we can design lots of detection sequences e.g. apoptotic nucleus (TUNEL-FITC filter---green), total nuclei (Hoechst-UV filter---blue), SMCs or macrophage cell type marker (SMC- α -actin antibody-Rhoadomin filter---red). As we know, the images are usually different from the scene under the microscope especially with traditional photo technique. With the aid of digital camera and computer program system, images can be stored and rechecked immediately. If the images are not taken well enough, repeated adjustments are possible.

In conclusions, with the aid of improved counting and quantification method as we designed, the variation of traditional investigation can be diminished, therefore, the quantitative apoptotic process can be represented more precisely.

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References

1. Isner JM, Kearney M, Bortman S, Passeri J. Apoptosis in human atherosclerosis and restenosis. *Circulation* 1995;91:2703-11.
2. Han DKM, Haudenschild CC, Hong MK, Tin kle BT, Leon MB, Liao G. Evidence for apoptosis in human atherosclerosis and in a rat vascular injury model. *Am J Pathol* 1995;147:267-77.
3. Hegyi L, Skepper JN, Cary NR, Mitchinson MJ. Foam cell apoptosis and the development of the lipid core of human atherosclerosis. *J Pathol* 1996;180:423-9.
4. Kockx MM, De Meyer GRY, Muhring J, Bult H, Bultink J, Herman A. Distribution of cell replication and apoptosis in the atherosclerotic plaques of cholesterol-fed rabbits. *Atherosclerosis* 1996;120:115-24.
5. Geng Y-J, Libby P. Evidence for apoptosis in advanced human atheroma: colocalization with interleukin 1 converting enzyme. *Am J Pathol* 1995;147:251-66.
6. Bjorkerud S, Bjorkerud B. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am J Pathol* 1996;149:367-80.
7. Henderson C. Aminoalkylsilane: an inexpensive, simple preparation for slide adhesion. *J Histochem* 1989;12:123-4.
8. Stary HC, Chandler AB, Dinsmore RE. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. *Circulation* 1995;92:1355-74.
9. Lucassen PJ, Chung WCJ, Vermeulen JP, van Lookeren Campagne M, van Dierendonck JH, Swaab DF. Microwave-enhanced *in situ* end labeling of fragmented DNA: parametric studies in relation to post mortem delay and fixation of rat and human brain. *J Histochem Cytochem* 1995;43:1163-71.
10. Strater J, Gunthert AR, Bruderlein S. Microwave irradiation of paraffin-embedded tissue sensitizes the TUNEL method for *in situ* detection of apoptotic cells. *Histochem* 1995;103:157-60.
11. Kerr JFR. Shrinkage necrosis: a distinct mode of cellular death. *J Pathol* 1971;105:13-21.
12. Wylie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980;284:555-6.
13. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119:493-501.
14. Bauriedel G, Hutter R, Schluckebier S, Welsch U, Prescott MF, Kandolf R, Luderitz B. Decreased apoptosis as a pathogenic factor in intimal hyperplasia of human atherosclerosis lesions. *Germ J Art Zeitschrift fur Kardiologie* 1997;86:572-80.
15. Whiteside G, Cougnon N, Hunt SP, Munglani R. An improved method for detection of apoptosis in tissue sections and cell culture, using the TUNEL technique combined with Hoechst stain. *Brain Res Protoc* 1998;2:160-4.