Short Communication

Dating Components of Human Atherosclerotic Plaques

Isabel Gonçalves, Kristina Stenström, Göran Skog, Sören Mattsson, Mihaela Nitulescu, Jan Nilsson

Rationale: Atherosclerotic plaques that give rise to acute clinical symptoms are typically characterized by degradation of the connective tissue and plaque rupture. Experimental studies have shown that mechanisms to repair vulnerable lesions exist, but the rate of remodeling of human plaque tissue has not been studied.

Objective: In the present study, we determined the biological age of different components of advanced human atherosclerotic plaques by analyzing tissue levels of $^{14}$C released into the atmosphere during the nuclear weapons tests in the late 1950s and early 1960s.

Methods and Results: Atherosclerotic plaques were obtained from 10 patients (age 46 to 80 years) undergoing carotid surgery. Different regions of the plaques were dissected and analyzed for $^{14}$C content using accelerator mass spectrometry. At the time of surgery, the mean biological age of the cap region was $6.4 \pm 3.2$ years, which was significantly lower than that of the shoulder region ($12.9 \pm 3.0$ years, $P < 0.01$), the interface toward the media ($12.4 \pm 3.3$ years, $P < 0.01$), and the core ($9.8 \pm 4.5$ years, $P < 0.05$). Analysis of proliferative activity and rate of apoptosis showed no signs of increased cellular turnover in the cap, suggesting that the lower $^{14}$C content reflected a more recent time of formation.

Conclusions: These results show that the turnover time of human plaque tissue is very long and may explain why regression of atherosclerotic plaque size rarely is observed in cardiovascular intervention trials. (Circ Res. 2010; 106:1174-1177.)

Key Words: atherosclerosis ▪ plaque ▪ dating ▪ $^{14}$C

Observations from animal models of atherosclerosis and human autopsy studies suggest that plaque development is initiated by accumulation and oxidation of lipoproteins in the arterial wall extracellular matrix, leading to activation of inflammation and intimal fibrosis.1 Advanced plaques with large lipid necrotic cores covered by thin fibrous caps are more susceptible to rupture and responsible for the development of the majority of clinical events.2 It has been postulated that the stability of atherosclerotic plaques depends on their ability to produce effective fibrotic repair responses.3 In humans, plaques develop silently over several decades, and it is not known to what extent they have a capacity to undergo continuous repair and remodeling. In this study, we aimed to clarify this by determining the biological age of individual components of human plaques. We used a method that allows assessment of human tissue turnover based on the incorporation of $^{14}$C released by nuclear weapons tests in the late 1950s and early 1960s, the so called “bomb-pulse.” Atmospheric levels of $^{14}$C increased rapidly after these detonations.4 When exposed to the atmosphere, $^{14}$C, like stable carbon, reacts with oxygen to form CO2 and is taken up by plants through photosynthesis. After 1963, atmospheric levels have subsequently dropped rapidly (Figure 1A), not because of radioactive decay ($^{14}$C half-life is 5730 years) but as a result of diffusion of $^{14}$CO2 into other parts of the ecosystem. Through dietary intake of $^{14}$C from plants, as well as animals that live on plants, the $^{14}$C concentration of human cells and tissues is generally expected to closely parallel that in the atmosphere at the time they were formed, with an average lag time of $\approx 1$ year.5,6 The potential of using the bomb-pulse to study replacement rates for human tissue was realized already at the beginning of the nuclear weapons test era.5,7,8 Refined measurement methods, using samples containing only a few tens of micrograms of carbon, have recently opened new possibilities and stimulated further applications of the technique.9 By determining $^{14}$C levels in different tissues, it has been shown that the formation of human cortical neurons,9 as well as the eye lens,10 almost entirely takes place around the time of birth and that human gallstones begin to form $\approx 10$ years before they give rise to symptoms.11 It has also been shown that $\approx 10\%$ of all fat cells12 and 0.5% to 1% of all of cardiomyocytes13 are turned over annually in humans.
Figure 1. Use of nuclear test $^{14}$C to calculate the time of formation of different regions in human plaque tissue. A, Atmospheric concentration of $^{14}$C in the northern hemisphere. Atmospheric testing of nuclear weapons during the late 1950s and early 1960s produced large amounts of $^{14}$C. The highest level was observed in 1963, when the specific activity of $^{14}$C in carbon dioxide was approximately twice the natural level (expressed in units of fraction modern, F$^{14}$C; F$^{14}$C is approximately equal to 1.0 before the nuclear weapons test). Since the test ban in 1963, atmospheric $^{14}$C-specific activity has decreased because of the uptake of CO$_2$ in oceans and biosphere and because of fossil fuel $^{14}$C-free CO$_2$ input. By analyzing the $^{14}$C content of tissues, the time of its formation can be determined. The uncertainty of the $^{14}$C measurement ($\pm$1 SD) results in a corresponding uncertainty in the age calibration. B, Mean biological age (years) of the different regions of the human atherosclerotic plaques (n=10). The box plot shows minimum, first quartile, median, third quartile, and maximum values. *P<0.05, **P<0.01.

Methods

We examined 10 atherosclerotic plaques from patients undergoing carotid surgery at the Malmö University Hospital during 2007 to 2009. The mean age of the patients was 65 (range, 46 to 80) years. Five of the patients had symptoms such as stroke (n=1), transient ischemic attacks (n=1), and amaurosis fugax (n=3), whereas 5 were referred to surgery because of a nonsymptomatic carotid stenosis of >70%. Informed consent was given by each patient. The study was approved by the regional ethical committee. After surgical removal, plaques were snap-frozen in liquid nitrogen. A 1-mm-thick transverse section of the plaque was used for histology, and a consecutive 1-mm-thick section was dissected to separate the fibrous cap, shoulders, core, and interface between the core and the underlying media of the plaque. The $^{14}$C content of isolated plaque fragments was quantified by accelerator mass spectrometry (AMS) using the

Non-standard Abbreviations and Acronyms

AMS | accelerator mass spectrometry
PCNA | proliferating-cell nuclear antigen
TUNEL | terminal deoxynucleotidyl transferase dUTP nick-end labeling

250 keV single-stage AMS facility (Lund University). Before these measurements, the carbon was extracted from the samples and converted into elemental carbon (graphite). This was achieved in a 2-step process involving the conversion of the carbon in the sample to CO$_2$, followed by reduction of the CO$_2$ to graphite. The graphite from each sample was pressed into a sample holder and placed in the ion source of the single-stage AMS facility, together with standard samples of known activity and background samples ($^{14}$C-free) processed in the same way. The results from the AMS measurements were converted to calendar years using the CalibBomb software and the Levin data set representative for Europe (Figure 1A). Histological sections were stained for apoptosis using TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) In Situ Cell Death detection kit POD (Roche Applied Science, Indianapolis, Ind), according to the instructions of the manufacturer. Proliferation was assessed by staining with mouse monoclonal [PC10] anti-human proliferating-cell nuclear antigen (PCNA) proliferation marker (ab29) (1:100, Abcam, Cambridge, UK) as primary antibody and incubated overnight at 4°C. Sections were subsequently incubated with biotinylated polyclonal rabbit antimouse F(ab)2 (E0413,1:200, DakoCytomation, Glostrup, Denmark) for 30 minutes and then with peroxidase-labeled streptavidin (Vectastain ABC-AP kit, Vector Laboratories, Peterborough, UK). Samples were scanned (ScanScope CS, Aperio), and stained areas measured with Biopix iQ 2.1.8 image analysis software. Values are presented as means±SD, and statistical significances were calculated using Mann–Whitney U test.

Results

The fibrous cap was identified as the most recently formed part of the plaque (Figure 1B). At the time of surgery, the mean biological age of the cap was 6.4±3.2 years, which was significantly lower than that of the shoulder (12.9±3.0 years, P<0.01), the interface toward the media (12.4±3.3 years, P<0.01), and the core (9.8±4.5 years, P<0.05). It should be noted that the data represent the mean age of the biological material in the sample and that the sample is likely to contain structures formed both before and after the indicated time point. The strategy used for dissection of the various plaque structures is exemplified in Figure 2. To clarify whether the lower $^{14}$C values in the cap region reflected a higher tissue turnover or a more recent time of formation, we determined PCNA and TUNEL (a marker of apoptotic cell death) in the fibrous cap and fibrous regions outside the cap. Proliferative activity expressed, as percentage of PCNA immunostained tissue area, was higher outside the cap (18.7±13.2% versus 8.6±5.4%, P<0.05), whereas there was no difference in TUNEL staining between cap and noncap regions (5.3±6.8% versus 4.76±1.8%).

Discussion

This study demonstrates that it is possible to determine an approximate biological age of human atherosclerotic plaque components using $^{14}$C analysis by AMS. The finding that the fibrous cap is the youngest part of advanced plaques suggests that this is the location where most of the growth of the lesion...
takes place or alternatively that the tissue turnover is higher in the fibrous cap than in the rest of the plaque. The latter possibility is in line with the notion that active proliferation and extracellular matrix synthesis by smooth muscle cells in the cap is required for maintaining plaque stability. However, our observation that proliferative activity and apoptosis were not increased in the cap argues against a higher tissue turnover as a possible explanation to the lower $^{14}$C in the cap in this study. These results demonstrate that the turnover of most of the atherosclerotic plaque tissue occurs at a very low rate. Results from lipid-lowering intervention trials have consistently shown that significant reductions in cardiovascular mortality occur without any or only marginal reduction of atherosclerotic plaque size. The finding that the age of most of the plaque tissue is $\approx 10$ years helps to explain these observations and suggests that, at least with existing therapies, reduction of plaque size is an unlikely response to treatment. This is also in line with the notion that statins primarily function by reducing plaque inflammation. The regression of atherosclerosis observed in experimental animal studies generally relates to relatively early lesions, and these findings cannot be directly extrapolated to humans.

There are several limitations of the present study that need to be considered. First, the study is based on relatively few patient samples. However, with the exception of the plaque obtained from a patient who had a major stroke at a young age, the results consistently demonstrate that the rate of tissue turnover in human atherosclerotic plaques is very low. Second, the uncertainty of AMS determination results in an uncertainty of up to $\pm 3$ years in the biological age of tissue sample (Figure 1). The uncertainty increases with more recently formed tissues because the difference in atmospheric $^{14}$C levels between years is lower. High-precision bomb-pulse dating may also be limited by small variations of $^{14}$C concentration in different environments and types of diets. Nevertheless, the effect of these variations is likely neither to exceed the uncertainty reported in this study nor to influence the conclusions. Finally, although the proliferation and apoptosis findings argue against an increased cellular turnover in the fibrous cap, they do not exclude the possibility of an increased turnover of noncellular proteins as an explanation for the younger biological age of the cap.

In conclusion, using $^{14}$C determination by AMS, we show that the biological age of human atherosclerotic plaque...
components varies between 5 and 15 years of age, with the cap being the youngest. It should be noted that this does not exclude that the plaque in it self is older and that the original plaque components have been replaced. The very slow turnover of atherosclerotic plaque tissue may help to explain why reduction of atherosclerosis has been difficult to achieve in cardiovascular intervention trials.

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Disclosures
None.

References

Novelty and Significance

What Is Known?
• Atherosclerotic plaques develop over many years.
• Tissue remodeling is important for maintaining plaque stability.
• The rate at which remodeling occurs in humans has not been studied.

What New Information Does This Article Contribute?
• The turnover of human plaque tissue is very slow, ranging from 5 to 15 years in different regions of the plaque.
• The fibrovascular cap is the youngest part of the plaque.

The ability to mount effective tissue repair responses is of critical importance for maintaining plaque stability, but the rate at which tissue turnover occurs in human atherosclerotic plaques has not been studied. We have taken advantage of the possibility to determine the time of formation of human tissue by analyzing the content of 14C released into the atmosphere during the nuclear weapons tests in the late 1950s and early 1960s. The 14C content of atherosclerotic lesions removed at carotid surgery was determined by accelerator mass spectrometry. The results demonstrate that most of the plaque tissue is formed 10 years or more before the time of surgery but that fibrous cap is several years younger. The very slow tissue turnover of atherosclerotic lesions may explain the relatively limited effect on plaque size observed in clinical intervention trials.
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