METHODS

Patients

The study was approved by the Mayo Foundation Institutional Review Board, and procedures followed institutional guidelines. Written informed consent was obtained before surgery. We studied 177 carotid plaques specimens from patients undergoing carotid endarterectomy, following a previously described procedure. The decision for the surgical intervention was based, following current guidelines, on neurological and neurosurgical examination, carotid ultrasound and/or MRI, both in symptomatic or progressive asymptomatic patients with extracranial carotid artery disease. Demographic data and detailed clinical history were obtained for each patient by chart review (Mayo Clinic Documents Browser and Mayo Integrated Clinical Systems (MICS) Last Word), with particular attention to carotid territory ischemic events. Carotid atherosclerosis clinical instability was defined subdividing the patients into symptomatic, in the presence of a cerebral ischemic event within six months prior to surgery, ipsilateral to the collected plaque, and asymptomatic, in the absence of ischemic events prior to surgery. Eligible clinical events to include patients in the symptomatic group were ischemic stroke, transient ischemic attack (TIA) and amaurosis fugax.

Plaque specimens

After surgery, plaques were halved at the site of the maximum plaque diameter. One half was fixed in formalin and embedded in paraffin for histology; the other half was immediately frozen at –80°C for subsequent tissue analysis. Plaque stability was evaluated by the tissue expression of matrix-metalloproteases (MMP)-2 and 9, macrophage infiltration, content of fibrous tissue and apoptotic rate.

Western Blotting
Samples were prepared and separated by electrophoresis as previously described\(^2\). Frozen samples were homogenized in lysis buffer and protein concentration of lysates calculated by Bradford assay (Bio-Rad, CA). Equal amount of proteins were diluted in lysis buffer and reducing SDS loading buffer and resolved in a 10% SDS-Page gel. Subsequently, electrotransfer to nitrocellulose membrane was performed and membranes were autoclaved for 30’ to increase the sensitivity of ubiquitin-conjugates detection\(^3\) and blocked in 5% fat-free dry milk. Membranes were then immunoblotted to detect ubiquitin-conjugates (rabbit polyclonal antibody, 1:1000, Sigma, St Louis, MO), ubiquitin (mouse monoclonal antibody, 1:500, Covance, Princeton, NJ), MMP-9 (rabbit polyclonal antibody, 1:5000, Chemicon International, Temecula, CA), MMP-2 (mouse monoclonal antibody, 1:7500, Chemicon International), NADPH-oxidase p67phox (rabbit polyclonal, 1:500, Santa Cruz Biotechnology Inc, Santa Cruz, CA), one of the principal sources of oxidative stress in human atherosclerosis\(^4\); protein loading control was evaluated using anti- β-Actin antibody (Sigma, 1:1000). Anti-rabbit (1:1000-1:5000, Amersham Life Sciences, IL ) or anti-mouse (1:1000, Amersham Life Sciences) antibodies conjugated to horseradish peroxidase were used as secondary antibodies, as appropriate. After developing with chemiluminescence (Pierce, IL)\(^2\) and exposing to X-ray film (Kodak, NY), signals were analyzed using ImageJ software (National Institutes of Health) and expressed as integrated density. For quantification of large ubiquitin-conjugates, the density of the membrane corresponding column was analyzed in each sample (see manuscript Figure 1A). To avoid the influence of slight differences in membranes conditions –e.g. different backgrounds- and to obtain comparable data among different runs, one same sample was repeated in every membrane and used to normalize the signal. On the basis of the signal obtained, we divided the whole population into quartiles of ubiquitin-conjugates content. Immunoblotting results are expressed as ratios to actin signal.

**Proteasome activity assay**
Chymotrypsin-like activity of the proteasome was assayed using a commonly available fluorimetric kit (APT280, Chemicon International), following company instructions. Briefly, frozen samples were homogenized in lysis buffer and protein concentration of lysates calculated by Bradford assay (Bio-Rad). Progressive dilutions of 20S purified proteasome were analyzed for standard activity curve. One hundred µg of proteins from each carotid plaque lysate and standard proteasome dilutions were incubated with the fluorogenic proteasome substrate Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC) for 60 minutes at 37°C, in the presence or absence of the proteasome inhibitor lactacystin. Generated fluorescence was read with a 380/460 nm filtered fluorometer (SpectraMax Gemini XPS, Global Medical Instrumentation Inc., Ramsey, MN). The proteasome activity was calculated subtracting the inhibited from the non-inhibited activity and expressed as generated nanomoles of AMC/milligram of protein/minute.

**Immunostaining for ubiquitin-conjugates, nitrotyrosine, macrophages and smooth muscle cells**

After deparaffinizing, hydrating, quenching endogenous peroxidase and blocking, carotid plaque sections were incubated overnight at 4°C with primary antibody and then for 1 h with anti-rabbit IgG secondary antibody (Dako A/S, Glostrup, Denmark). Diaminobenzidine was used as chromogen. For double staining procedures, after staining with the first primary antibody, slides were blocked (Doublestain block, Dako) and incubated with the second primary antibody (1 hour), followed by alkaline phosphatase conjugated secondary antibody (Dako) and Vector Blue (Dako, blue label) or Fast Red (Dako, red label) substrate. All sections were counterstained either with Hematoxylin or Nuclear Fast Red (Sigma). Normal rabbit or mouse immunoglobulin fraction was substituted to primary antibodies as negative immunostaining control.

Primary antibodies used: anti-ubiquitin-(conjugates) (1:100, Sigma), anti-3-nitrotyrosine (1:250, Sigma), anti-α-SMA (1:500, Dako), and anti-CD68(1:500, Dako).
Stained sections were then mounted and visualized under microscope (Olympus, Leeds Precision Instruments) and pictures were taken with an imaging program (SPOT Advanced 3.3, Diagnostic Instruments Inc). For the quantification of smooth muscle cells and macrophage, the percent area of the specimen stained with the corresponding color was calculated by the use of a image analysis computer software (MetaMorph, Meta Imaging Series 4.6).

**Expression of ubiquitin-conjugates in smooth muscle cells and macrophages**

Representative slices of whole plaques were prepared for double immunofluorescence by deparaffinizing and hydrating. Double immunofluorescence was also applied to single cells after dispersion and adherence of cells to glass coverslip. Briefly, after removing fat, adventitia, and blood residues from carotid plaque specimens, the remaining tissue was incubated with a collagenase II dissociation solution, at 37°C in a shaker bath. After repeated trituration, sieving and washing, cells were plated in Hanks’s solution on glass coverslips and fixated with methanol:acetone 1:1.

After overnight incubation with the two primary antibodies, a mixture of secondary donkey anti-rabbit FITC and anti-mouse Texas Red conjugated antibodies (Jackson Immunoresearch Laboratories, Baltimore, PA) was applied to whole plaques or cell dispersions slices. The first primary antibody was anti-ubiquitin(conjugates) (1:100, Sigma) and the second primary antibodies were either anti-α-SMA (1:500, Dako) or anti-macrophage CD-68 (1:500, Dako). Observation of fluorescence was performed using a Zeiss LSM-510 confocal laser scanning microscope (Carl Zeiss, Inc., Oberkochen, Germany).

**TUNEL Staining**

Apoptosis was evaluated in-situ by the TUNEL procedure, using a commercially available kit (Apoptag® Peroxidase In Situ Apoptosis Detection Kit, Chemicon) and following manufacturer’s instructions. Briefly, after deparaffinizing and rehydrating, Proteinase K (20 µg/mL) was applied to
tissue sections for 10 minutes at room temperature. Following rinsing, permeabilization of the tissue was obtained by incubation with 0.05% triton X–100 in 0.05% sodium citrate for 5 s. Endogenous peroxidase was blocked with 2% H2O2 for 15 minutes. Subsequently, slides were incubated with equilibration buffer (5 min at RT), terminal deoxynucleotidyl transferase (TdT) enzyme (60 min at 37°C), stop/wash buffer (10 min at RT), protein block (Dako, 7 min at RT). Antidigoxin-peroxidase was then applied (30 min at RT) and followed by DAB substrate. A second labeling of macrophage CD68 or smooth muscle cell α-SMA was also performed as described above. Slides were finally counterstained, dehydrated and mounted. Rat regressive mammary gland tissue was used as a positive control for apoptosis (Intergen Company, Purchase, New York). Omission of TdT enzyme from the labeling procedure served as a negative control.

Sirius Red Staining
The interstitial collagen content of carotid plaques was evaluated by Sirius red. Specimen sections were deparaffinized, rehydrated and incubated with 0.1% Sirius red in saturated picric acid for 60 minutes. After incubation in 1% acetic acid for 30 minutes and rinsing, slides were counterstained in hematoxylin, differentiated in acid alcohol solution, rehydrated and mounted. Slides were visualized under both bright-field and polarized light microscope, and pictures taken with identical exposure settings for all sections. The content of collagen type I and II, identified by birefringence under polarized light5, was evaluated as percent of the plaque area.

Statistics
For clinical data, Western blotting and proteasome activity variables were compared by use of the Student t-test or the x² test. Comparison among multiple groups was performed by ANOVA followed by Tukey-Kramer post-hoc analysis. Correlation was calculated with Pearson product moment. Data are expressed as percentage or mean±SE for continuous variables and by percentage for qualitative variables. Statistical significance was assumed for P<0.05.


