APPENDIX

Tissue preparation for immunostaining

Sections were deparaffinized in xylene-ethanol solution and incubated in 2N hydrochloride (HCl) (for 5-bromodeoxyuridine [BrdU] stain) for 30 minutes. After washing with normal saline solution, sections were incubated with 1% hydrogen peroxide (H$_2$O$_2$) in methanol for 30 minutes. After washing with phosphate-buffered saline solution (PBS), sections were incubated with goat serum (1:200 dilution in PBS) for 20 minutes. Sections were then incubated with mouse anti-BrdU monoclonal antibody (Becton Dickinson Immunocytometry Systems; 1:50 dilution in 1% BSA in PBS) or with anti-α–smooth muscle (α-SM) actin mouse anti-human monoclonal antibody (Cruz Biotechnology; at a concentration of 20 μg/mL), respectively. After PBS rinse, sections were incubated with biotinylated anti-mouse immunoglobulin G antibody for 30 minutes with the avidin-biotin complex (ABC) method (Vector Laboratories,) according to the manufacturer’s protocol. Sections were counterstained with hematoxylin before counting.

Preparation for dioxyuridine triphosphate nick end–labeled (TUNEL) staining

Sections were deparaffinized, rehydrated, and incubated in 1% H$_2$O$_2$ in methanol for 30 minutes. After PBS washing, DNA fragmentation was detected with an in situ apoptosis detection kit (Apop@Tag Plus Peroxidase; Intergen), according to the manufacturer’s protocol. The sections were incubated with biotinylated secondary antibody as required according to the manufacturer’s protocol (ABC method). Sections were counterstained with hematoxylin.

Reverse transcriptase polymerase chain reaction

Complementary DNA synthesis. Contaminated DNA was removed by treatment with deoxyribonuclease I (Invitrogen). One microgram of total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) to cDNA. Primers. Rat vascular endothelial growth factor (VEGF), KDR, and platelet-derived growth factor–β (PDGF–β) primers were designed from the rat cDNA of VEGF-D (NM031836; forward primer 5′-caagtcgccactcttgcagga-3′ and reverse primer 5′-gctgctttcatctctgtcct-3′) and PDGF–β (L40991; forward primer 5′-cagcagctgcggggtaatc-3′ and reverse primer 5′-cgatgaggttccgcgagat-3′). These primers had been tested in rats to enable amplification of specific polymerase chain reaction (PCR) products. β-Actin primers were also designed from rat cDNA of β-actin (L40991; forward primer 5′-tcttgccctactctgcc3′ and reverse primer 5′-ggggccgactctgact-3′) and tested in rat to obtain specific PCR products.

Standard curve. The PCR products amplified by the primers were purified and quantified. One nanogram of each PCR product was stepwise diluted to serial picogram concentrations. Reverse transcriptase (RT) PCR was performed with the protocol below. RT-PCR results from each primer pair were analyzed, and standard curves were created.

Polymerase chain reaction. RT-PCR was performed with the GeneAmp 7700 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. Reactions were performed in a 20 μL volume with 0.8 μmol/L primers and 1 μg cDNA and SYBR Green PCR Master Mix (Applied Biosystems). A typical protocol included 2-minute incubation at 50°C for optimal AmpErase UNG enzyme activity, followed by 10 minutes at 95°C for activation of AmpliTaq Gold DNA polymerase, followed by 40 cycles of 15-second denaturation at 95°C and 1-minute annealing and extension at different annealing temperatures determined by each pair of primers. For confirmation of amplification specificity, RT-PCR products were loaded on 2% agarose gel in 0.5 X TBE buffer for gel electrophoresis. The RT-PCR products from each primer pair were subjected to standard curve analysis and normalized against β-actin expression.

Gelatin gel zymography

Samples were ground in liquid nitrogen, homogenized in buffer (50 mmol/L Tris-HCl, 10 mmol/L calcium dichloride [CaCl$_2$], 2M guanidine HCl, 2.5% Triton X-100), incubated on ice, and centrifuged at 4°C for 15 minutes. Supernatant was dialyzed against 50 mmol/L Tris-HCl and 2.5% Triton X-100 overnight at 4°C. Samples were normalized for total protein content. For detection of MMP-2 and MMP-9 (gelatinase A and B, respectively), equal amounts of tissue extract protein (20 mg), assayed with the BCA protein assay (Bio-Rad), were loaded on each lane and run in parallel in 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis gels containing 1% gelatin. Gels were washed with Triton X-100 (2.5%) to remove SDS, then incubated overnight (37°C) in developing buffer (50 mmol/L Tris base and 10 mmol/L CaCl$_2$). Zones of lysis were visualized after staining the gels with 0.5% Coomassie blue R-250. Densitometric analysis of lytic bands for MMP-2 and MMP-9 was performed by public domain software NIH Image version 1.61.