Ribonuclease Inhibitor Regulates Neovascularization by Human Angiogenin

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EXPERIMENTAL PROCEDURES

Materials. *Escherichia coli* strain BL21(DE3) and the pET22b(+) expression vector were from Novagen (Madison, WI). *E. coli* strain TOPP 3 (Rif^r [F′ *proAB lacI*^{q}*ZΔM15* Tn10 (Tet^r) (Kan^r)]), which is a non–K-12 strain, was from Stratagene (La Jolla, CA). Enzymes for DNA manipulations were from Promega (Madison, WI) or New England BioLabs (Beverly, MA). Oligonucleotides and 6-FAM–dArUdAdA–6-TAMRA, where 6-FAM refers to 6-carboxyfluorescein and 6-TAMRA refers to 6-carboxytetramethylrhodamine, were from Integrated DNA Technologies (Coralville, IA). All other chemicals and biochemicals were of commercial grade or better, and were used without further purification.

Instruments. Absorbance measurements were made with a Cary model 50 spectrophotometer (Varian, Sugarland, TX). Fluorescence was measured with a QuantaMaster1 photon-counting spectrophotometer from Photon Technology International (South Brunswick, NJ). Microscopy was performed with a LSM 510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NJ).

Preparation of Proteins. Plasmids that direct the production in *E. coli* of wild-type RNase A, G88R RNase A, and wild-type ANG were described previously (*1, 2*). Site-directed mutagenesis of the plasmid encoding wild-type ANG with the oligonucleotide AGGCCAGGGAGA**TCTTCT** ATGTAGCTT was used to replace both Gly85 and Gly86 with arginine residues (reverse complement in boldface type).

Ribonucleases and porcine RI were prepared as described previously (*1–3*), except that wild-type ANG and its G85R/G86R variant were refolded in the presence of 0.10 M NaCl instead of 0.5 M arginine. To eliminate contaminating ribonucleolytic activity, all buffers were treated with diethyl pyrocarbonate (DEPC), and all tubing and glassware were treated with RNase Erase (MP Biomedicals, Aurora, OH) and rinsed extensively with DEPC-treated deionized distilled H_2O prior to column chromatography. Ribonuclease concentrations were determined by UV spectroscopy using $\varepsilon = 0.72$ (mg/mL)$^{-1}$·cm^{-1} at 277.5 nm for RNase A (*4*) and G88R RNase A, and $\varepsilon = 0.83$ (mg/mL)$^{-1}$·cm^{-1} at 280 nm for wild-type ANG and its G85R/G86R variant (*2*). Ribonucleases were dialyzed extensively against phosphate-buffered saline (PBS) prior to use in all RI-binding assays as well as all cell-based assays.

Zymogram Electrophoresis. Zymogram electrophoresis was performed as described previously to confirm that purified wild-type ANG and its G85R/G86R variant were free from contaminating ribonucleolytic activity (*2, 5*). Briefly, ANG samples were subjected to SDS–PAGE in which the reducing agent was omitted from the sample buffer and the gel was copolymerized with poly(cytidylic acid) (0.5 mg/mL), which is a substrate for ANG and RNase A. After electrophoresis, SDS was removed from the gel by washing (2 × 10 min) with

10 mM Tris–HCl buffer at pH 7.5, containing 2-propanol (20% v/v). Ribonucleases were renatured by washing (2 × 10 min) with 10 mM Tris–HCl buffer at pH 7.5, and then washing (15 min) with 0.10 M Tris–HCl buffer at pH 7.5. The gel was stained for 1 min with 10 mM Tris–HCl buffer at pH 7.5, containing toluidine blue (0.2% w/v), which stains high-M_r nucleic acids. Finally, the gel was destained in deionized distilled H_2O for 10 min. Protein bands possessing ribonucleolytic activity appear clear in a dark background (Figure S1).

Enzymatic Activity. The catalytic activity of ribonucleases was measured with the fluorogenic substrate 6-FAM–dArUdAdA–6-TAMRA (*6*). Cleavage of this substrate results in a ~200-fold increase in fluorescence intensity (excitation at 492 nm; emission at 515 nm). Assays were performed at 23 °C in 0.10 M MES–NaOH buffer at pH 6.0, containing NaCl (0.10 M), 6-FAM–dArUdAdA–6-TAMRA, and enzyme. Data were fitted to the equation: $k_{cat}/K_M = (\Delta I/\Delta t)/\{(I_f - I_0)[E]\}$, where $\Delta I/\Delta t$ is the initial velocity of the reaction, I_0 is the fluorescence intensity prior to the addition of enzyme, I_f is the fluorescence intensity after complete hydrolysis with excess RNase A, and [E] is the ribonuclease concentration.

Assays of Ribonuclease Inhibitor Binding. The fluorescence of fluorescein-labeled A19C/G88R RNase A (fluorescein–RNase A) decreases by ~15% upon binding to porcine RI (*7*). The affinity of RI for G85R/G86R ANG was determined by a competition assay in which RI was allowed to bind to G85R/G86R ANG in the presence of fluorescein–RNase A (*7*). Briefly, G85R/G86R ANG (1 nM–2 μM) and fluorescein–RNase A (50 nM) were incubated in 2.0 mL of PBS for 30 min at 23 °C. The fluorescence intensity (excitation at 491 nm; emission at 511 nm) was measured before and after the addition of porcine RI (50 nM). Values of K_d for the complex between RI and G88R RNase A were determined as described previously (*7*).

Rabbit Cornea Micropocket Assay. Neovascularization was assessed with a micropocket assay, as described previously (*8*). Briefly, a polyhydroxyethylmethacrylate hydrogel pellet containing vehicle, wild-type ANG (10 μg), or G85R/G86R ANG (10 μg) was implanted in a micropocket made 3 mm from the limbus in the transparent corneal stroma of New Zealand white rabbit eyes. Four eyes were treated in each group. In a blind experiment, the 12 rabbit eyes were examined daily under slit-lamp biomicroscopy by two observers. Pictures of corneal neovascularization were taken with a zoom photographic slit lamp (Takagi® model SM-50F; Nakano, Japan). Corneal neovascularization was measured directly from slides using an image analyzer consisting of a CCD camera (SONY® CCD TR-900, Japan) coupled with digital analyzer software (Optima® version 5.1.1). Angiogenic activity was defined as the number of newly developed vessels multiplied by the length of vessels from the limbus, and was measured on postoperative days 3, 7, 10, and 14. Length values were scored according to the following scale: zero for vessels <0.3 mm, one for 0.3–0.6 mm, two for 0.7–0.9 mm, and three for >1.0 mm. When one vessel branched into several vessels, the longest vessel was used for a representative score. The scores of the two observers were summed, and the mean was used as the final score.

Histological Examination. Animals were euthanized by general anesthesia on day 14 after pellet implantation. Tissue samples were enucleated and fixed in aqueous formaldehyde (4% v/v). Sections of 4 μm were obtained, stained with hematoxylin and eosin, and observed with a light microscope.

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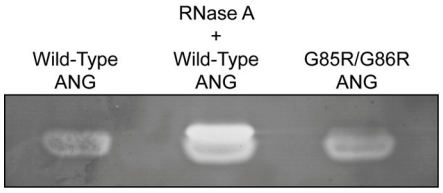

FIGURE S1: Zymogram electrophoresis of wild-type ANG and its G85R/G86R variant. Lane 1, Wild-type ANG (8 μg); lane 2, wild-type ANG (8 μg) and RNase A (0.25 ng); lane 3, G85R/G86R ANG (8 μg).