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Assessing the effects of threonyl-tRNA synthetase on angiogenesis-related responses

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ABSTRACT

Several recent reports have found a connection between specific aminoacyl-tRNA synthetases and the regulation of angiogenesis. As this new area of research is explored, it is important to have reliable assays to assess the specific angiogenesis functions of these enzymes. This review provides information about specific *in vitro* and *in vivo* methods that were used to assess the angiogenic functions of threonyl-tRNA synthetase including endothelial cell migration and tube assays as well as chorioallantoic membrane and tumor vascularization assays. The theory and discussion include best methods of analysis and quantification along with the advantages and limitations of each type of assay.

1. Introduction

Angiogenesis is defined as the formation of new blood vessels from a pre-existing vasculature that occurs during both development and in tissues responding to injury, hypoxia and/or nutrient stress. The regulation of angiogenesis is tightly controlled by substances secreted by the cells experiencing stress, the responding endothelial and mural cells, as well as the surrounding blood cells including immune cells and platelets [1]. Our laboratory has recently discovered that threonyl-tRNA synthetase (TARS) is among a subgroup of aminoacyl-tRNA synthetases that is secreted by hypoxic cells and stimulates an angiogenic response [2,3]. The methods described below outline both *in vitro* and *in vivo* methods used to identify the angiogenic properties of TARS including troubleshooting tips and advantages and limitations of each individual assay. Because we found that TARS secretion underlies the angiogenic response, most of these assays use purified recombinant TARS applied to the extracellular space which will be considered within the discussion along with future applications of angiogenic assays in the study of non-canonical functions of aminoacyl-tRNA synthetases.

2. Materials and methods

2.1. Expression and purification of human threonyl-tRNA synthetase

The assays described below use recombinant amino-terminal His_6-tagged human TARS (also known as ThrRS) expressed and purified from *E. coli*. The quality of the purified protein is important for consistent results in the angiogenesis assays and the following protocol produces TARS that has confirmed activity in both aminoacylation and angiogenesis assays [2].

(1) *E. coli* Rosetta™ 2(DE3)pLysS competent cells (EMD) are transformed with the TARS plasmid (pET28a hctThrRS) as in Bovee et al. [4]. Transformant cultures are grown in terrific broth including 100 mg/ml kanamycin and 100 mg/ml chloramphenicol at 37 °C to a cell density of $A_{600} = 0.6$. Expression of TARS is induced with 1 mM isopropyl 1-thio-β-D-galactoside overnight at 15 °C. *Note:* after induction, cell pellets can be frozen, which improves the cell lysis and protein recovery.

Abbreviations: TARS, threonyl-tRNA synthetase; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; CAM, chorioallantoic membrane; PECAM-1, platelet endothelial cell adhesion molecule; MVA, microvascular area.

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(2) The resulting bacterial pellets are lysed by sonication in buffer A (20 mM potassium phosphate buffer pH 8.0, 100 mM KCl, 35 mM imidazole, and 5 mM β-mercaptoethanol) and cleared by centrifugation at 17,050×*g* for 30 min. Nucleic acids are precipitated by the addition of protamine sulfate to a final concentration of 0.3% and removed by centrifugation.
(3) The TARS-rich supernatant is loaded onto a HisTrap™ FF column (GE Healthcare) equilibrated with buffer A and eluted by an imidazole gradient of 35–250 mM in buffer A over 20 column volumes in 5 ml fractions. The fractions containing TARS are identified by separating 10 μl of the fractions by SDS-PAGE followed by GelCode™ Blue staining (Thermo Scientific). TARS-containing fractions are then pooled, and dialyzed into buffer B (100 mM potassium phosphate buffer pH 6.8 and 5 mM β-mercaptoethanol).
(4) The partially purified TARS is then loaded onto a CHT-Tricorn Hydroxyapatite column and eluted over 20 column volumes by using a gradient of buffer B to buffer C (500 mM potassium phosphate pH 8.0 and 5 mM β-mercaptoethanol). TARS-containing fractions are determined by SDS-PAGE as above, pooled, and dialyzed into buffer D (10 mM HEPES pH 8.0, 100 mM KCl, 2.5 mM β-mercaptoethanol, and 40% glycerol). Protein concentration is determined by A_{280} and aliquots are then stored at −20 °C. *Note:* Prior to dialysis, save 0.5 ml of buffer D (storage buffer) to use as a protein blank and as a control in angiogenesis assays. This second purification step is critical for generating consistently pure TARS for experiments. Stock concentrations of TARS are made at 100 μM to generate final experimental concentrations in the nM range.

2.2. In vitro angiogenesis assays

Endothelial responses are key to the initiation and progression of angiogenesis [5]. The *in vitro* angiogenesis assays assess the impact of compounds on the capacity of endothelial cells to migrate and form tubes which indicates the propensity to break down extracellular matrix and assemble in a directed fashion. These assays are simple to conduct and relatively easy to quantify, thus are often the first assays used to screen for angiogenic potential. In our assessment of TARS effects on angiogenesis, we use a model of low passage (passage 2–6) cultured human umbilical vein endothelial cells (HUVECs) that are grown on plates coated with 0.2% gelatin and maintained in endothelial growth media (EGM™-2, Clonetics).

2.2.1. Transwell migration assay

The transwell migration assay uses a modified Boyden chamber that consists of a porous membrane between 2 chambers. Cells are applied to the upper chamber and cells that cross the membrane are counted to determine the migration effects [6]. Vascular endothelial growth factor (VEGF) is used as a positive control and migration should be initially examined at several time points between 2 and 24 h. The challenge is getting the appropriate control media conditions that allow survival but do not induce migration; below is the protocol that produced consistent results in measuring the impact of TARS on endothelial cell migration.

(1) HUVECs are serum-depleted overnight in low-serum EGM™-2 (0.2% FBS). Cells trypsinized, counted and $5X10^4$ cells are plated in the upper chamber of 0.2% gelatin-coated 24-well 8 μm Transwell™ inserts (Corning) containing control media (90% endothelial basal media (EBM™-2), 10% EGM™-2). The lower chamber contains control media plus the test compounds (i.e. 50 ng/ml VEGF, 100 nM TARS protein).
(2) Culture chambers are incubated for 4 h and then fixed in 10% formalin. Cells present on the top of the membrane are removed with a cotton swab. Cells that migrate to the bottom of the membrane are stained with 10 μg/ml DAPI solution (Roche) and imaged using a 4x objective on the Olympus IX70 inverted microscope (Olympus). DAPI-stained nuclei are quantified using ImageJ software as described below.

2.2.2. Endothelial tube formation assay

Another important *in vitro* method that indicates angiogenic activity is the endothelial cell tube formation assay. This assay uses HUVECs that are plated in the presence of extracellular matrix components (Matrigel™), and fetal bovine serum is used as a positive control to stimulate tube formation [7,8]. The advantage of this assay is that it measures formation of endothelial cell tubes in an environment which more closely mimics conditions during new blood vessel formation. For experiments with TARS, a relatively short incubation period is used to reduce background tube formation effects [2].

(1) The 48-well plates are first coated with Matrigel™ (Basement Membrane Matrix Growth Factor Reduced; BD Biosciences). *Important Note*: The Matrigel™ solution is solid at -20 °C, liquid at 0 °C, and solidifies into a gel at 25 °C. The solution cannot be thawed quickly; it must be thawed overnight on ice at 4 °C and approximately 100 μl aliquoted into wells using pipet tips that have been stored at −20 °C. Work quickly and change tips if the solution becomes thick. Avoid bubbles by setting the pipet at 110 μl and not ejecting the entire amount. Place the plate in the culture incubator for 30 min while preparing the cells.
(2) To prepare the cells, start with low-passage HUVECs grown on a gelatin-coated 10 cm plate. Cells are trypsinized, removed from the plate in a solution of trypsin neutralizing solution and EGM™-2, and then collected by centrifugation. The HUVEC pellet is gently, but fully, resuspended in 300 μl serum-free EBM™-2 media (Lonza) and cells are counted. Sterile microfuge tubes are prepared that contain the desired components (EBM media only (negative control), EGM™-2 media + 2% FBS (positive control) and EBM™-2 media + 200 nM TARS). Cells are added such that there are 5.5×10^4 cells/well in a total of 200 μl solution/well. Samples should be prepared in duplicate or triplicate.
(3) Plates are incubated for 4–6 h in a 37 °C incubator, stopping the reaction when tubes are visible in the positive control wells. The tubes are then fixed with 10% formalin, stained with Oregon Green 488 phalloidin (1:150; Molecular Probes), and imaged using an inverted light microscope. *Note:* when aspirating cells after the incubation period, take care when suctioning to prevent aspiration of the gel matrix. Imaging is optimal in the 48-well format as the small diameter of the 96-wells results in distortion of tubes for imaging. The number of tube branches (in pixels) are quantified using the Angiogenesis Analyzer plug-in on ImageJ software as described below [9].

2.3. In vivo angiogenesis assays

In vivo assays have the obvious advantage of the physiological environment under which new vessels are formed, but the disadvantage of difficulties in application of test compounds and variability in quantification. For measurements of TARS effects on angiogenesis, we utilized the chicken chorioallantoic membrane (CAM) assay and a syngenic mouse ovarian tumor model.