

New Role for tRNA and Its Fragment Purified From Human Urinary Bladder Carcinoma Conditioned Medium: Inhibition of Endothelial Cell Growth

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Abstract The growth of endothelial cells is necessary for angiogenesis, which in turn is required for later steps of tumor progression. In an attempt to purify new modulators of endothelial cell growth from the conditioned medium of human urinary bladder carcinoma cells, we isolated a small and stable oligonucleotide containing 10 to 16 bases. This oligonucleotide inhibited the growth of endothelial cells *in vitro* and was identified as a fragment of transfer RNA (tRNA). When unfractionated bovine tRNA was added to the cell culture, it specifically inhibited growth of endothelial cells, but not smooth muscle cells, bovine kidney cells, 3T3 fibroblasts, and several cancer cell lines. In contrast, ribosomal RNA, total yeast RNA, and single nucleosides from tRNA hydrolysate had no effect. These results demonstrate a new role for tRNA and its fragment as a selective endothelial cell inhibitor *in vitro*. *J. Cell. Biochem.* 76:109–117, 1999. © 1999 Wiley-Liss, Inc.

Key words: human bladder carcinoma; endothelial cell inhibitor; transfer RNA

Several types of RNA and DNA oligonucleotides have been found to inhibit tumor cell growth. Probably, the best known are antisense oligonucleotides—single stranded RNA or DNA molecules, that block translation of target mRNA by complementary hybridization [Crooke, 1992]. Antisense oligonucleotides against certain growth factors, growth factor receptors, and oncoproteins were shown to inhibit tumor cell growth *in vitro* and *in vivo* [Curcio et al., 1997; Lavrovsky et al., 1997]. Antisense oligonucleotides also potentiate the effects of conventional chemotherapy in several tumor models [De Luca et al., 1997; Kornmann et al., 1998]. Furthermore, inhibition of cancer cell proliferation could be achieved by transfection with triplex-forming oligonucleotides [Neidle, 1997] as well as ribozymes—small RNA

molecules capable of catalytically cleaving themselves or other RNAs [Persides, 1997]. Although most currently tested active RNAs and DNAs are synthetic compounds, ribozymes, antisense oligonucleotides, and triple-helical structures occur also in nature [Curcio et al., 1997]. Accordingly, there are a growing number of functions for ribo and deoxyribonucleic acids, which differ from their originally assigned roles of vehicles of genetic information. We therefore asked whether biologically active forms of RNA or DNA could be purified from extracellular fluids. Media collected from cultures of cancer cells have been successfully used in the past for the purification of stimulators and inhibitors of cell proliferation [Shing et al., 1993; O'Reilly et al., 1997]. Conditioned medium of bladder carcinoma cell line 5637 is known to be particularly rich in modulators of cell growth, both inhibitors, and stimulators. They include, among others, hematopoietic colony-stimulating factors [Zinzar et al., 1985; Gabrilove et al., 1986; Welte et al., 1985], interleukin-1 [McNiece et al., 1989], and leukemia inhibitory factor [Gascan et al., 1990]. This medium also inhibits growth of endothelial cells *in vitro* as well as formation of capillary-like tubes in a collagen gel sandwich

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assay (R. Montesano, unpublished observations), suggesting that it contains potential anti-tumor compounds, which act through inhibition of angiogenesis [reviewed in Folkman, 1995]. Leukemia inhibitory factor appeared to be one of these molecules [Pepper et al., 1995]. Here, we report the isolation of a new type of endothelial cell inhibitor from the conditioned medium of human bladder carcinoma cells. This inhibitor is a small and stable RNA oligonucleotide that we characterized as a fragment of transfer RNA (tRNA). Furthermore, we extended our studies on unfractionated, commercially available tRNA and determined that this tRNA also has inhibitory activity on the growth of endothelial cells.

MATERIALS AND METHODS

Conditioned Medium

The human urinary bladder carcinoma (UBC) cell line 5637 was purchased from American Type Culture Collection (ATCC). Cells were grown in 900 cm² roller bottles containing 125 ml of DMEM supplemented with 5% fetal bovine serum (FBS) and 1% glutamine-penicillin-streptomycin (GPS) at 37°C in 5% CO₂. After 4 days, the medium was replaced with serum-free medium. The serum-free medium was harvested 72 h later and stored at 4°C.

Ribonucleic Acids

ubcRNA was purified as described in the following section. Unfractionated bovine liver transfer RNA (R4752) and ribosomal RNA (R5502) were purchased from Sigma. RNA mix (G3191) was purchased from Promega. Single stranded DNA (P1680) with 24 nucleotides was purchased from Sigma. Double stranded DNA PCR product of 380 bp was a gift from Dr. Sui Huang of Children's Hospital, Boston.

Enzymes

DNase-free RNase (1119915) was purchased from Boehringer Mannheim. RQ1 RNase-free DNase (M6101) and S1 nuclease (M5761) were purchased from Promega. Mung bean nuclease (M8202K) was purchased from Epicenter Technologies. Nuclease P1 (N8630), phosphodiesterase I (P6903), and alkaline phosphatase (P4252) were purchased from Sigma.

Purification of Endothelial Inhibitory Activity From the Conditioned Medium of UBC Cell Line

The purification was carried out using sequentially Bio-Rex 70 Resin (Bio-Rad Laboratories, Hercules, CA), DEAE Sepharose Fast Flow (Pharmacia Biotech, Piscataway, NJ), and HPLC C₄ reversed phase column (SynChrom, Inc., Lafayette, IN). Conditioned medium was first applied to a Bio-Rex column (90 cm × 5 cm) equilibrated with 50 mM NaCl, 10 mM Tris-HCl (pH 7.0). The flow-through was collected and applied to a DEAE column (50 cm × 4.5 cm) equilibrated with 50 mM NaCl, 10 mM Tris-HCl (pH 7.0). The DEAE column was subsequently washed with the equilibration buffer and then eluted with 400 ml of 0.2 M NaCl, 10 mM Tris-HCl followed by 400 ml of 0.5 M NaCl, 10 mM Tris-HCl. Fractions of 20 ml were collected and an aliquot of each was applied to cultured endothelial cells. Fractions that inhibited endothelial cell [³H]thymidine incorporation and proliferation were pooled and loaded on HPLC C₄ column (100 mm × 4.6 mm) equilibrated with H₂O containing 0.1% trifluoroacetic acid (TFA). The column was eluted with a gradient of acetonitrile in 0.1% TFA at 0.5 ml/min. Fractions of 1 ml were collected and evaporated to dryness by vacuum centrifugation. An aliquot of each fraction dissolved in 0.1 ml of distilled water was applied to endothelial cell cultures. Inhibitory activity was further purified by two subsequent cycles on the C₄ column.

Bovine Capillary Endothelial Cell Proliferation and [³H]thymidine Incorporation Assay

Bovine capillary endothelial cells were cultured as previously described [Folkman et al., 1979]. For proliferation assay, cells were plated (20,000 cells/ml) in DMEM + 10% BCS + 1% GPS, onto gelatinized 24-well culture plates (0.5 ml/well), and incubated at 37°C in 10% CO₂ for 24 h. The medium was replaced with 0.5 ml of DMEM + 5% BCS + 1% GPS and the test sample applied. After 72 h, cells were trypsinized, resuspended in Hematall (Fisher Scientific, Pittsburgh, PA), and counted with a Coulter counter. For [³H]thymidine incorporation assay, cells (10,000 cells/ml) were plated onto gelatinized 96-well culture plates (0.2 ml/well), and incubated at 37°C in 10% CO₂ for 24 h. The test sample was then applied. After 6 h, 1 μCi of [³H]thymidine (NEN, Boston, MA) per well was added, and 18 h later, cells were dis-

persed in trypsin. The amount of [³H]thymidine incorporated into DNA was determined using a Microbeta scintillation counter (Wallac, Turku, Finland).

Cell Proliferation Assay for Non-Endothelial Cells

Bovine aortic smooth muscle cells (SMC), bovine kidney cells (BKC), 3T3 fibroblasts, Lewis lung carcinoma (LLC) cells, urinary bladder carcinoma (UBC) cells, and B16 melanoma (B16M) cells were maintained at 37°C in 10% CO₂. Optimal conditions for cell proliferation assays were established for each cell type. Bovine calf serum was used for the SMC, BKC and 3T3 cells. Fetal bovine serum was used for the tumor cell types. Cell suspension (20,000 cells/ml for SMC, B16M and UBC cells; 15,000 cells/ml for 3T3 fibroblasts; 10,000 cells/ml for BKC and LLC cells) was plated onto 24-well culture plates (0.5 ml/well), and incubated at 37°C in 10% CO₂ for 24 h. The media was then replaced with 0.5 ml of DMEM + 5% BCS + 1% GPS and samples applied. After 72 h, cells were dispersed in trypsin, resuspended in Hematall (Fisher Scientific, Pittsburgh, PA), and counted with Coulter counter.

Analysis of ubcRNA, tRNA, and rRNA by HPLC and Gel Electrophoresis

The active ubcRNA samples purified from HPLC C₄ column were analyzed on 15% SDS-PAGE ready gel (Bio-Rad laboratories, Hercules, CA) and 4% agarose ready gel (FMC, Rockland, ME). ubcRNA was visualized with silver stain and ethidium bromide stain on SDS-PAGE and agarose gels, respectively. Amino acid composition of the sample was determined after complete hydrolysis and microsequencing was performed [Lane et al., 1991] by automated Edman degradation on an ABI model 477A protein sequencer (Applied Biosystems, Foster City, CA).

Enzymatic hydrolysis of RNA to single ribonucleosides was carried out with nuclease P1, phosphodiesterase I and alkaline phosphatase as previously described [Crain, 1990]. The hydrolysate was analyzed with a HPLC reversed phase C₁₈ column (150 × 4.6 mm) (Rainin, Woburn, MA). The C₁₈ column was equilibrated with buffer A (2.5% [v/v] methanol in 0.01 M NH₄H₂PO₄, pH 5.1). After loading of the RNA hydrolysate, major and modified nucleosides were eluted by buffer A for 45 min, followed

by buffer B (10% [v/v] methanol in 0.01 M NH₄H₂PO₄, pH 5.1) for 30 min [Davis et al., 1979]. The elution was performed at room temperature with a flow-rate of 0.5 ml/min. UV spectra of individual modified nucleosides were determined with Beckman DU 640 spectrophotometer.

RESULTS

In the attempt to purify new modulators of tumor growth from the conditioned medium of human urinary bladder carcinoma cells, we used a combination of chromatographic techniques, which allows the purification of both proteins and nucleic acids. The conditioned medium was first applied to a Bio-Rex column. The anti-proliferative activity was found in the flow-through, which was then subjected to DEAE chromatography. The active material recovered from the DEAE column by elution with 0.5M NaCl in 10mM Tris (pH 7.0) was further purified with three cycles of a HPLC C₄ column. The purified material inhibited both [³H]thymidine incorporation into DNA and proliferation of bovine capillary endothelial cells *in vitro* (Fig. 1). This material revealed a single band with silver stain on SDS-PAGE (Fig. 2A). Analysis of this single band material on 4% agarose gel revealed a band which was stained with ethidium bromide (Fig. 2B) and comprised nucleic acids containing between 10 and 16 nucleotides. This material was sensitive to digestion with DNase-free RNase, S1 nuclease (Fig. 3), and mung bean nuclease, but not with RQ1 RNase free DNase (data not shown). These results indicated that the purified material contains single stranded RNA. We therefore named it ubcRNA. Further analysis of the ubcRNA preparation revealed that besides RNA it also contains amino acids with a predominance of glycine (data not shown). No N-terminal amino acid sequence was identified indicating that either there is no peptide molecule or that it is blocked at the N-terminus. The identification of glycine and other amino acids in the ubcRNA fraction raises the question as to whether amino acids play a role in the endothelial inhibitory activity of tRNA. Given that the commercial bovine tRNA is active but does not contain significant amounts of amino acids, it seems unlikely that amino acids, by themselves, play an important role. However, the potential role of aminoacyl-tRNA synthetase which combines amino acid to

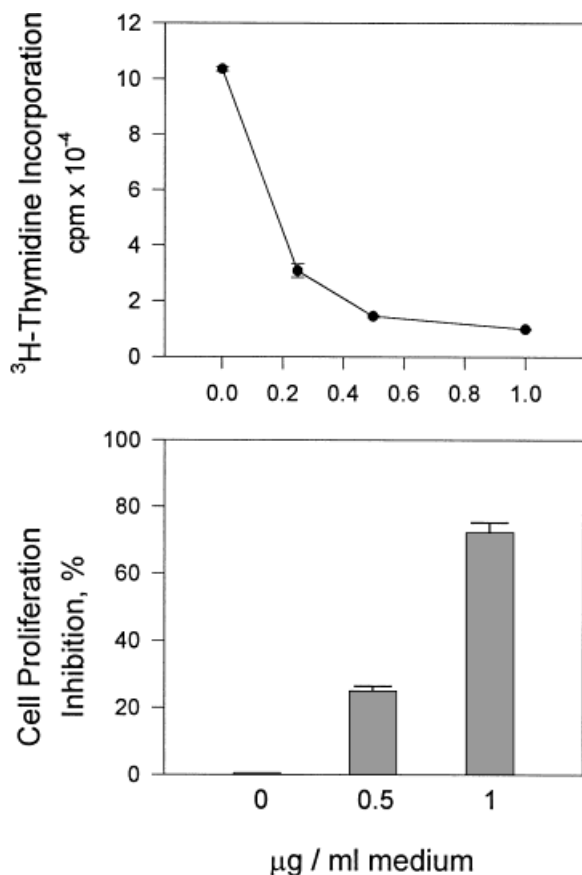


Fig. 1. Effects of ubcRNA on bovine capillary endothelial cells. Inhibition of DNA synthesis (upper panel) and cell number (lower panel) by ubcRNA ($\mu\text{g/ml}$) in endothelial culture medium.

tRNA in the growth of endothelial cell, certainly is worth further investigation.

Tumor growth is often associated with high levels of tRNA-specific modified nucleosides in the extracellular fluid [Borek and Kerr, 1972]. In order to assess the relationship between ubcRNA and tRNA, we analyzed 20 μg each of ubcRNA, tRNA and ribosomal RNA (rRNA) on a HPLC C_4 column. As shown in Figure 4, tRNA (A) and ubcRNA (B), but not rRNA (C) yielded a peak at 23-min (20–25% acetonitrile). In addition, tRNA and ubcRNA were individually hydrolyzed to single nucleosides, and analyzed on HPLC C_{18} column (Fig. 5). This experiment demonstrated that all of the modified nucleotides identified in the sequence of ubcRNA could also be found in the bovine unfractionated tRNA. By comparison with published HPLC C_{18} elution profiles of calf liver tRNA [Gehrke and Kuo, 1989], we found that the major modified nucleosides found in ubcRNA were pseudo-

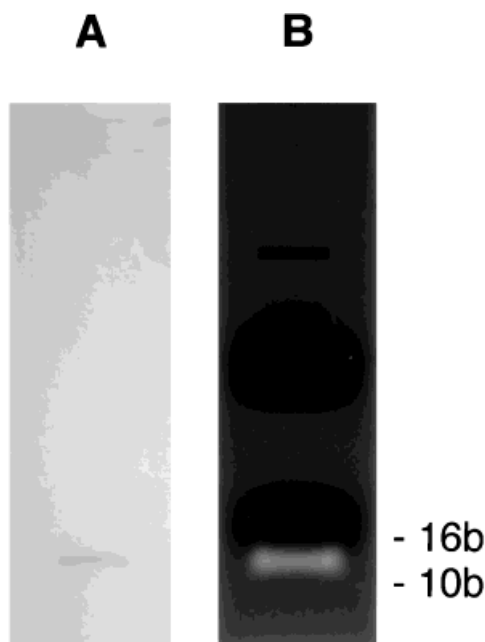


Fig. 2. Analysis of purified ubcRNA on 15% SDS-PAGE with silver stain (A) and 4% agarose gel with ethidium bromide stain (B).

uridine (ψ), 2'-O-methylpseudouridine (ψm) and 2'-O-methylguanosine (Gm). This was confirmed by the analysis of their individual UV spectral properties [Gehrke and Kuo, 1989] (data not shown). Taken together, these data strongly suggest that ubcRNA is a fragment of tRNA. Interestingly, this inhibition was selective for endothelial cells. Neither bovine aortic smooth muscle cells, nor five other cell types were inhibited by comparable concentrations of tRNA (Fig. 6). UbcRNA has also been found to be selective for endothelial cells, but not for other tested cell lines such as bovine smooth muscle and human bladder carcinoma cells.

The structural integrity of tRNA was not required for its inhibitory activity, since ubcRNA as well as a commercial tRNA fragmented by heating at 95°C (data not shown) also inhibited endothelial cells. In order to test the possibility that the inhibitory activity was due to the individual nucleosides, we compared the effect of whole tRNA and its hydrolysate on endothelial cells. Figure 7 shows that in contrast to tRNA molecules, single nucleosides composing these molecules have no inhibitory activity. Furthermore, incubation of endothelial cells with 10 μM dipyrindamole, a blocker of transport of nucleosides and nucleotides into these cells [Lemmens et al., 1996] did not prevent the

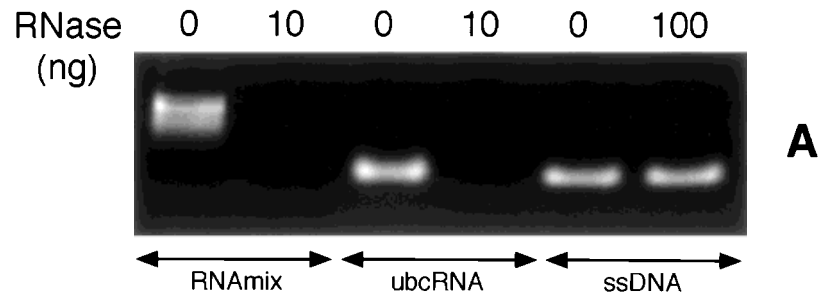


Fig. 3. Digestion of ubcRNA with RNase (A) and S1 nuclease (B). Two μg of each sample were incubated at 37°C for 30 min with varying concentrations of DNase-free RNase (A), or for 50 min with varying concentrations of S1 nuclease (B). Products of reactions were visualized on 4% agarose gel containing ethidium bromide with UV light. RNase did not hydrolyse control DNA, but did digest control RNA mix and ubcRNA. Single strand-specific S1 nuclease hydrolyzed single stranded control DNA and ubcRNA, but not double stranded control DNA.

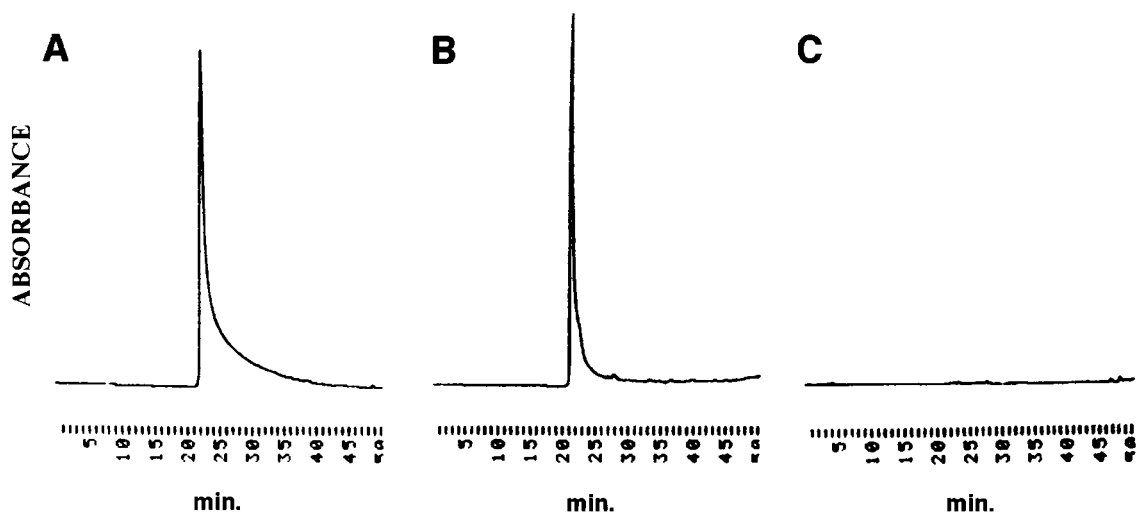
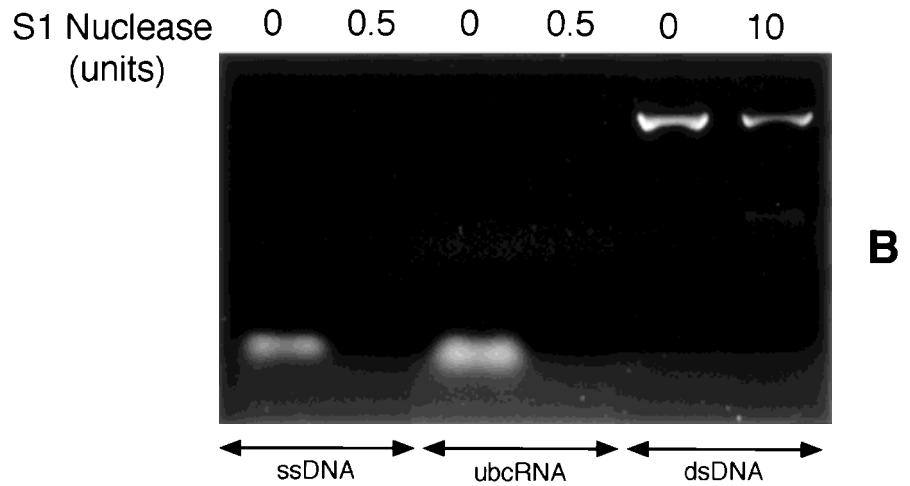


Fig. 4. Analysis of 20 μg of RNA on HPLC reversed phase C_4 column. A: ubcRNA. B: Bovine liver tRNA. C: rRNA. Ribosomal RNA and some degraded fragments of tRNA appeared to be trapped in the column due to the increasing concentration of acetonitrile. Only ubcRNA and tRNA-derived ubcRNA-like molecules were solvable and eluted identically at 23-min (20–25% acetonitrile).

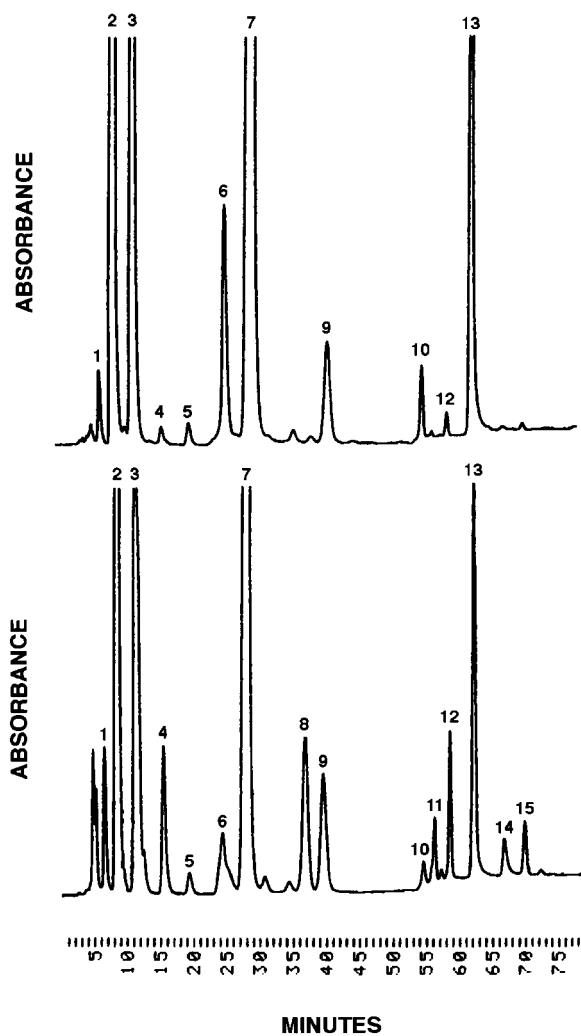


Fig. 5. Analysis of nucleosides from hydrolysates of ubcRNA (upper panel) and tRNA (lower panel) on HPLC reversed phase C_{18} column. 1: Pseudouridine; 2: Cytidine; 3: Uridine; 4: 5-Methylcytosine; 5: 7-Methylguanosine; 6: Inosine; 7: Guanosine; 8: 2'-O-Methyluridine; 9: 2'-O-Methylpseudouridine; 10: 2'-O-Methylguanosine; 11: 1-Methylguanosine; 12: N²-Methylguanosine; 13: Adenosine; 14: N², N²-Dimethylguanosine.

tRNA-mediated inhibition of BCE cells, although it inhibited the incorporation of [³H]thymidine (data not shown). These experiments demonstrate that the effect of tRNA is mediated through a nucleotidic sequence.

DISCUSSION

The use of RNA and RNA-derived materials for cancer diagnosis and treatment has a long history. Fragments of tRNA and modified ribonucleosides from extracellular fluids have been found to be markers of carcinogenesis [Borek et al., 1977; Speer et al., 1979; McEntire et al.,

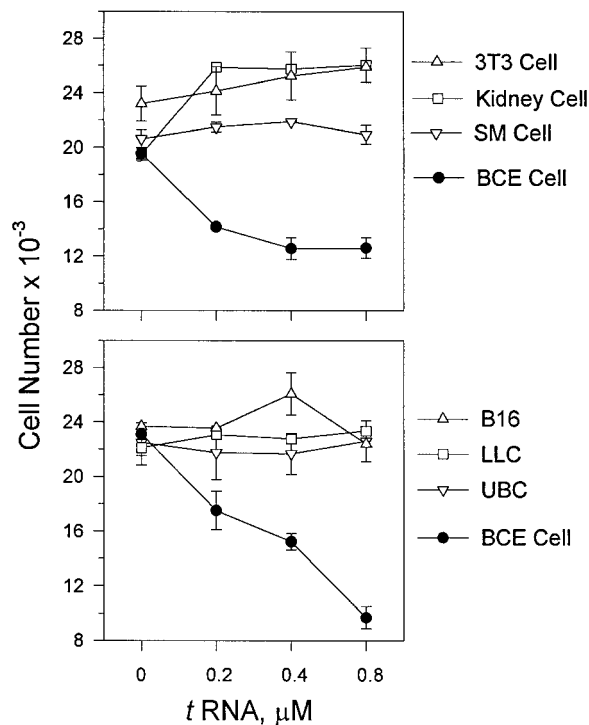


Fig. 6. Effect of tRNA on proliferation of different cell types.

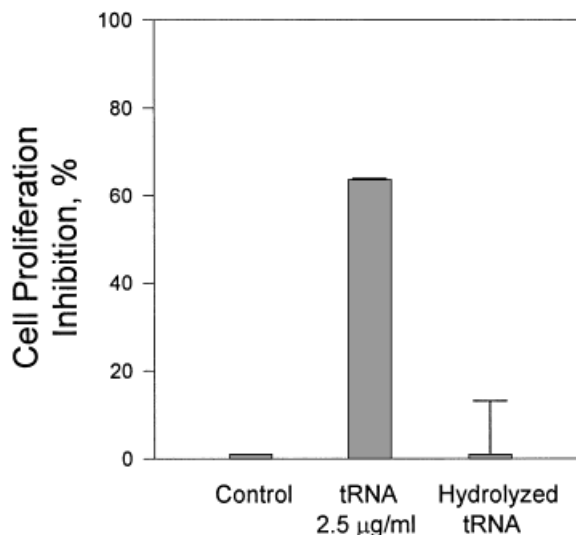


Fig. 7. Effect of tRNA (2.5 μ g/ml) and its hydrolysate on BCE cell proliferation. Control: PBS.

1989]. Further, double stranded RNAs were proposed for use in anticancer therapy, due to their stimulatory effect on the antiproliferative function of interferon [Larson et al., 1969; Yamamoto et al., 1979; Hubbell et al., 1984, 1992]. More recently, antisense RNA, ribozymes, and triplex-forming oligonucleotides emerged as po-

tential modulators of oncogene expression [Curcio et al., 1997]. Here, we report that tRNA and its fragment (ubcRNA) contain a selective cytostatic activity against endothelial cells *in vitro*, which raises the possibility that these molecules may be potential candidates for inhibitor of angiogenesis. To our knowledge, this is the first report of extracellular tRNA being involved in the modulation of cell growth. A previous study has shown that transfection of kidney cells with purified tRNA from the same quiescent cells can cause up to 11% inhibition of their proliferation [Kanduc, 1996]. In our study, tRNA or ubcRNA were not transfected into the cells but simply added to the culture medium. Therefore, these RNAs could face both extra- and intracellular targets. They could bind to growth factors, or growth factor receptors as in the case for certain synthetic oligos [Jellinek et al., 1994; Rockwell et al., 1997]. Fragments of tRNA could also cross the cell membrane, and inhibit protein synthesis by competing with intact tRNA for binding to ribosomes.

The finding that fragments of tRNA can be purified from extracellular medium of urinary bladder carcinoma cell line raises the interesting question of their provenance. They could be either excretory products, or products of dead cells. It is tempting to speculate that since many tumors secrete RNases, including a tRNA-specific angiogenin [Saxena et al., 1992], tRNA or its fragments could be part of a physiologically relevant regulatory system in the extracellular fluids.

Our purification protocol allowed us to isolate both antiproliferative proteins and nucleic acids from the UBC cell conditioned medium. The subsequent use of HPLC C₄ column enabled us to purify an active, DNA-free, RNA-containing fraction, ubcRNA. Unlike rRNA, both ubcRNA and tRNA yielded an identical peak when analyzed on HPLC. Also, they display comparable inhibitory activity towards endothelial cells, while rRNA does not. Finally, the resolution of ubcRNA hydrolyzate on HPLC C₁₈ column generated an elution profile compatible with that of tRNA. HPLC C₁₈ chromatography of hydrolyzed ubcRNA revealed the presence of several modified nucleosides such as ψ m and Gm in its sequence. ψ m and Gm are rare modified nucleosides, present only in some tRNA species. ψ m localizes to the anticodon stem of human gly-

cine tRNA isoacceptor [Gupta et al., 1980], and Gm is part of D loop in human serine tRNA [Capone et al., 1985]. Interestingly, these two amino acids are the most abundant in the ubcRNA preparation. Testing the anticodon domain of glycine isoacceptor and the D loop domain of serine tRNA for the inhibitory activity against endothelial cell proliferation will be important for further understanding of this new role of tRNA.

In order to rule out the possibility that the antiproliferative activity of ubcRNA comes from other material than tRNA sequences, we tested the antiproliferative activity of purified bovine tRNA. Incubation of endothelial cells with HPLC purified and endotoxin-free tRNA resulted in total growth inhibition, confirming that ubcRNA activity resided in its tRNA-derived sequences. The direct comparison between specific activities of ubcRNA and tRNA was not possible in our study, because of their provenance from different species (human bladder carcinoma for ubcRNA and bovine liver for whole tRNA). Indeed it seems that species specificity is important for the inhibitory effect of tRNA. For example, tRNA isolated from wheat germs has three times lower activity than bovine tRNA towards bovine endothelial cells. Furthermore, bovine tRNA was found to have only moderate effect on the inhibition of tumor growth in mice (results not shown). The purification of tRNA from mouse source and more ubcRNA are needed to further assess the activity of tRNA and its fragments in the murine tumor model.

Among different cell types tested, tRNA was highly selective for endothelial cells. Not only tumor and established cell lines, but also primary cell culture of smooth muscle cells were not inhibited by equal concentrations of tRNA. The explanation for this selectivity lies certainly in the nature of the target(s) of tRNA, which remain to be identified. So does the minimal sequence of tRNA required for its antiproliferative effect. We have established that upon hydrolysis to single nucleosides, the inhibitory potential of tRNA is lost. On the other hand, the entire, intact structure of tRNA molecule is not required for this effect. This suggests that a short segment of tRNA, possibly including ψ m or Gm is responsible for the antiproliferative effect of tRNA on endothelial cells.

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