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Regulation of β1 integrin expression in endothelial cells by chimeric $tRNA^{Val}$ ribozyme

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To downregulate expression of the β1 integrin subunit in endothelial cells, plasmid encoding the ribozyme cassette containing hammerhead ribozyme flanked at the 5′ terminus by $tRNA^{Val}$ and at the 3′ terminus by constitutive transport element sequences was constructed. When used to transfect immortalized human endothelial cell line EA.hy 926, it selectively blocked the synthesis of the β1 integrin subunit and thus inhibited migration and proliferation of the cells. Thus, this construct may be a valuable tool to control the proangiogenic phenotype of stimulated endothelial cells.

Keywords: integrins, hammerhead ribozyme, cell migration, β1 integrin subunit

INTRODUCTION

Integrins are the most important family of cell surface receptors that mediate cell–matrix interactions (Hynes, 1992). Cell migration requires the coordinated activation and deactivation of integrins (Lauffenburger & Horwitz, 1996). As a cell migrates across a matrix, integrins at the leading edge of the cell adhere to the substrate (Huttenlocher *et al*., 1996). At the same time, receptors at the trailing edge of the cell detach from the substrate to allow the cell to progress forward (Palecek *et al*., 1998). Thus, during the sprouting process of angiogenesis, integrin affinity states are constantly being modulated. The αvβ3-integrin has been shown to play a critical role in angiogenesis, but several studies also delineate the essential contribution of β1 integrins in endothelial morphogenesis (Bloch *et al*., 1997; Eliceiri & Cheresh, 1999). There is experimental evidence that enhanced β1 integrin expression plays a role in the increased adhesion of cells (Leong *et al*., 2002). Given the importance of integrins in the pathogenesis of many human diseases, integrin receptors are very attractive targets for therapeutic intervention. Thus, regulation of integrin expression by synthetic oligodeoxyribonucleotides (Koivisto *et al*., 1994), DNAzymes (Cieslak *et al*., 2002; 2003) or viral-mediated infection of endothelial cells to express antisense RNA endogenously (Dallabrida *et al*., 2000) has been suggested as an alternative method to inhibit angiogenesis *in vivo*.

In this study, we characterize a hammerhead ribozyme that was designed to downregulate β1 integrin subunit expression in cells. For this purpose we constructed a chimeric $tRNA^{Val}$-ribozyme according to a scheme recently described by Koseki *et al*. (1999). This ribozyme is synthesized in the cell nucleus and then exported to the cytoplasm, i.e. to the same cellular compartment as β1 mRNA. We demonstrated that cells that expressed the specifically designed ribozyme showed a significantly decreased β1 mRNA level indicating that this approach can be efficient in controlling β1 integrin expression in migrating cells.

MATERIALS AND METHODS

Materials. Plasmid for the endogenously generated $tRNA^{Val}$-driven and CTE helicase-associated

Abbreviations: CTE, constitutive transport element; D-MEM, Dulbecco's modification of Eagle's medium; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phospahte-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; Rz, ribozyme.

hammerhead ribozyme (pUC-KE-tRNA-CTE) was kindly supplied by Professor Kazunari Taira (Department of Chemistry and Biotechnology, University of Tokyo, Japan). EA.hy 926 cells were kindly provided by Professor Cora-Jean S. Edgell from the University of North Carolina (Chapel Hill, USA).

Chemicals. The Reverse Transcription System and enzymes for PCR and ligation were from Promega. D-MEM and all supplements were obtained from Gibco. LipofektAMINE Reagent and LipofektAMINE Reagent PLUS were from Invitrogen Life Technologies. Total RNA Mini kit was from A&A Biotechnology.

Construction of plasmids for ribozyme expression. As a target for the hammerhead ribozyme we have chosen the 5′-GUC-3′sequence between nucleotides 594 and 596 from the AUG start codon of β1 integrin subunit mRNA. We used the *mfold* program 3.0 version (Zuker *et al*., 1999) for selection of the cleavage site of β1 integrin subunit mRNA and designing the sequence of the specific ribozyme. The hammerhead ribozyme contains the cleavage loop and two substrate recognition arms, whose 10-nucleotide-long sequences are complementary to the β1 integrin subunit mRNA sequences at the 3′ and 5′ sides from the chosen 5′-GUC-3′ triplet.

The template oligonucleotide coding for the hammerhead ribozyme cgg ttc gaa acc ggg cac tac aaa aac caa cTC TGT TCC AAC TGA TGA GGC CGA AAG GCC GAA ACT TTT TAC AAA Agg tac ccc gga tat ctt ttt ttc tat cgc gtc gac ct (113 nt) was synthesized chemically. The sequences of the hammerhead catalytic core and the substrate recognition arms are given in capital letters. The template was PCR amplified with the usage of primers: upper — 5′ TCC CCG GTT CGA AAC CGG GCA CTA C 3′, lower — 5′ CTG CAG GTC GAC GCG ATA GAA AAA AA 3′. Then the product of amplification was digested with *Kpn* I and Csp45 I endonucleases and ligated with pUC-KE-tRNA-CTE which also had been digested with the same endonucleases. After digestion both DNAs were extracted with a phenol/chloroform mixture, precipitated with 95% ethanol and the pellet was washed with 70% ethanol. The ligation was performed for 15–18 h at 15°C, using T4 DNA ligase.

Competent *Escherichia coli* JM109 cells, prepared using the calcium chloride method (Sambrook *et al*., 1989), were transfected with the ligation mixture by the heat shock method. The next stage was a quick analysis of the size of the PCR amplification product performed on bacterial lysates (PCR colony method). The plasmid DNAs giving a PCR product of the expected size (295 bp) were then obtained in a mini prep scale from *E. coli* JM109 cells and their sequences were analyzed with the PCR primer P7 (5′ CGC CAG GGTT TTCC CAGT CAC GAC 3′) on an ABI Prism 310 Genetic Analyzer sequencer.

Cell culture, transfection and cell lysis. We used human endothelial cell line EA.hy 926, derived by fusion of human umbilical vein endothelial cells with the human lung carcinoma cell line A549. Cells were cultured in D-MEM supplemented with 0.45% glucose, L-glutamine, streptomycin, penicillin and 10% FCS. Cells were grown in a monolayer, the culture flasks were maintained at 37°C in a humidified atmosphere of 5% CO_2. Transfection of cells at 70–80% confluence with milligrams of the ribozyme-containing plasmid or pUC-KE-tRNA-CTE expression cassette, as a negative control, was carried out for 6 h using 20 μg of LipofektAMINE reagent according to the manufacturer's protocol. After transfection the medium was changed to complete D-MEM and cells were incubated for up to 60 h.

Quantitation of $tRNA^{Val}$ ribozyme produced in endothelial cells. Cells were washed twice with PBS and treated with trypsin, suspended in D-MEM and centrifuged. Total RNA was isolated from the pellets using Total RNA Mini kit according to the manufacturer's protocol. Then RNA was precipitated with 96% ethanol and 10 M potassium acetate (added to a final concentration of 1 M) at –70°C. The amount of isolated RNA was quantified spectrophotometrically.

The presence of the ribozyme RNA was confirmed by relative semi-quantitative reverse transcription and PCR using GAPDH as an internal standard. The total RNA (2.5 μg) was used for the reverse transcription reaction according to the M-MLV reverse transcriptase manufacturer's protocol. cDNA was amplificated in 30 cycles of PCR reaction using *Taq* polymerase. To monitor the level of the ribozyme mRNA we used the pair of primers previously used for template amplification.

Determination of β1 integrin subunit mRNA. To detect β1 integrin subunit mRNA the same protocol of RT-PCR and PCR was used with the following primers: 5′ GGT GCA ATG AAG GGC GTG TTG G 3′ and 5′ GGA AAT GGT GTT TGC AAG TGT CG 3′**.** The final products of PCR were separated by electrophoresis in 8% polyacrylamide gels and then densitometrically analyzed.

"Wound healing-like" assay (cell migration). EA.hy 926 cells were grown on 24-well, 1% gelatin-coated microplates. Transfection of cells at 80–90% confluence with the ribozyme-encoding plasmid or the empty plasmid pUC-KE-tRNA-CTE was carried out for 3 h using LipofektAMINE Reagent PLUS according to the manufacturer's protocol. Untreated cells served as a control. Then the monolayer was wounded in the middle of each well using a micropipette tip and images were recorded immediately after the wounding (time zero), 4, 12, 24, 36 and 48

h later. Cell migration (movement into the denuded area) was observed at a magnification of 400×, photographed and quantified using image analysis of two fields of view in which nine distances of denuded area were chosen at random.

The lesion area in each field of view was measured and using the data from time 0 (T_0 wound area) the wound area was then converted to give mean percentage recovery from two identically treated plates (%R) using the equation:

$$\%R = \left[1 - \frac{\text{wound area at } T_t}{\text{wound area at } T_0}\right] \times 100$$

Where: T_0 is the wounded area at 0 h; T_t is the wounded area (4–48) h post-injury.

RESULTS AND DISCUSSION

The formation of new blood vessels is critical to the development of normal tissues as well as growth of solid tumors and to a large extent depends on specific molecular interactions between vascular cells and the extracellular matrix (Hanahan & Folkman, 1996; Bouck *et al*., 1996). Aberrant angiogenesis is also a key process for progress of many disorders including atherosclerosis (O'Brien *et al*., 1994), diabetic retinopathy (Hammes *et al*., 1996) and restenosis (Panda *et al*., 1997). Currently, two groups of integrin receptors are known to regulate the adhesive interactions during angiogenesis, namely the β1 and β3 subfamilies. The β1 subfamily of integrins seems to be a particularly good target for suppressing angiogenesis, since α5β1 and α2β1 are major adhesive receptors in human angiogenic blood vessels promoting endothelial cell morphogenesis, migration, and tube formation (Eliceiri & Cheresh, 1999; Bayless *et al*., 2000).

Ribozyme strategy seems to be very convenient to inhibit reversibly the expression of oncogenes and growth factor genes and thus to control angiogenesis (Szala *et al*., 2003). However, the efficiency of in-cell synthesized ribozymes depends not only on their secondary structure and effective export from the nucleus to the cytoplasm, but also on the concentration and colocalization with the target RNA. *Trans*-acting ribozymes such as hammerhead ribozymes are able to cleave RNA substrates in a sequence-specific manner. They cleave target mRNA in the most efficient way after the triplet 5′-GUC-3′ or 5′-CUC-3′ (Koseki *et al*., 1999). A suitable secondary structure of hammerhead ribozymes is required to assure their correct folding and catalytic activity. Keeping in mind the significant role which endothelial cells play in angiogenesis, in the present study we focused on generation of a ribozyme which could be used to control expression of β1 integrins. We produced such a construct, pUC-KE-tRNA-CTE-Rz (Rz, ribozyme), which has a complex structure (Fig. 1). For this purpose we have chosen the 5′-GUC-3′ sequence, 594–596 nucleotides from the AUG start codon, to be attacked in β1 mRNA. Using a novel expression cassette with a CTE-aptameric sequence, problems related to the target RNA secondary structure, particularly its involvement in intramolecular bonding, were overcome (Warashina *et al*., 2001). The expression cassette (pUC-KE-tRNA-CTE) contained: (i) the $tRNA^{Val}$ gene sequence at the 5′ end of the ribozyme sequence, which would thus mimic a 3′-immature tRNA molecule which is effectively exported to the cytoplasm after recognition by Exportin-t. In this way effective ribozyme export from the nucleus to the cytoplasm, where the target RNA is localized, could be promoted (Kuwabara *et al*., 2001; Kato *et al*., 2001). (ii) **C**onstitutive **t**ransport **e**lement sequence (CTE) at the 3′ end of the ribozyme — RNA binding to intracellular RNA helicases, which should destroy any secondary structure in the target RNA, thus facilitating its efficient binding and subsequent cleavage by the ribozyme (Warashina *et al*., 2001). Sequence analysis of the obtained construct showed that it differed from the designed one only in the position C^{109}. However, even with this mutation, the secondary structure of the fragment of the $tRNA^{Val}$ ribozyme and its activity seemed to be retained. This was because the nucleotides of the catalytic core essential for the cleavage activity (C^{99}, U^{100}, G^{101}, A^{102}, G^{104}, A^{105}, G^{110}, A^{111}, A^{112}) and the arms were not changed (Shimayama *et al*., 1995; Rola & Kuzmak, 2001).

When the hammerhead ribozyme complementary to the β1 integrin subunit mRNA was cloned into pUC-KE-tRNA-CTE plasmid and used to transfect EA.hy 926 cells, its intracellular expression could be observed for up to 40 h (Fig. 2). In this experiment, the level of β1 mRNA and Rz mRNA were analyzed by RT-PCR using total RNA isolated from EA.hy 926 cells as a template. We observed reduced expression of β1 integrin subunit mRNA in the course of time (Fig. 2). As it is seen also in Fig. 2, the level of the ribozyme mRNA in the cells increased from the time of transfection until it reached a maximum at 28 h. The level of Rz mRNA expression was dependent on the amount of pUC-KE-tRNA-CTE-Rz DNA used for the transfection (Fig. 3). The utility of a tRNA-driven hammerhead ribozyme encoded in pUC-KE-tRNA-CTE plasmid was recently demonstrated in respect to efficient inhibition of β-secretase (BACE1) gene expression in HEK293 cells (Nawrot *et al*., 2003).

To test the consequence of reduced expression of β1 integrins mRNA in endothelial cells, we

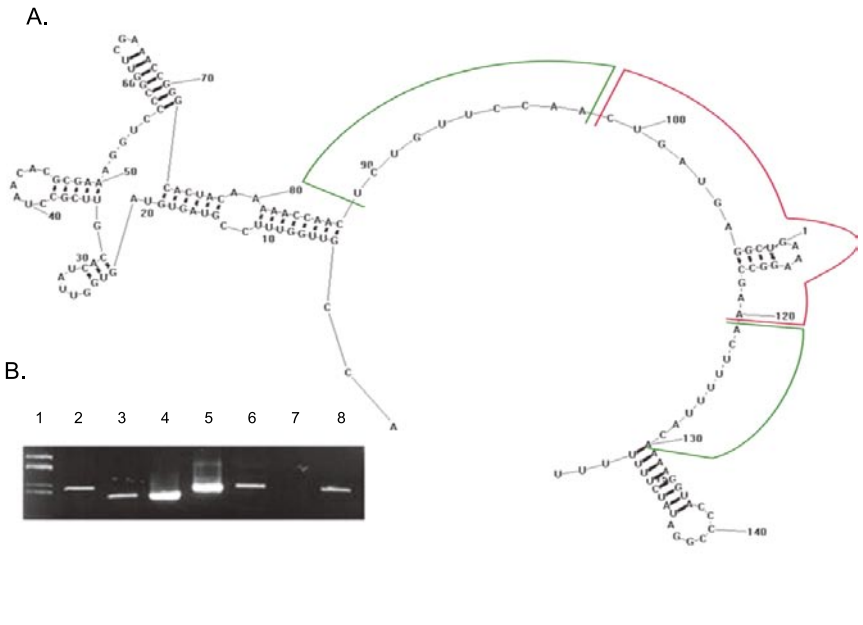

Figure 1. Construction of ribozyme to β1 integrin subunit mRNA.
A. Computer-generated secondary structure of the designed $tRNA^{Val}$ ribozyme. The hammerhead ribozyme contains the cleavage loop (red) (from C^{99} to A^{120}, 22 nt) and two substrate recognition arms (green) (from U^{89} to A^{98} and from A^{121} to A^{130}, respectively), whose 10-nucleotide sequences are complementary to the β1 integrin subunit mRNA sequence. **B.** RT-PCR performed directly on bacteria lysates (PCR colony) resulted in selection of 295 bp products which were then identified by restriction analysis and sequencing. Lane 1: a size marker, pUC digested with *Hae*III, Lines 2, 5, 6 and 8, clones taken for further sequencing, their length corresponds to 298 bp (pUC/*Hae*III). Lines 3 and 4, negative results of ligation, corresponding to 267/257 bp (pUC/*Hae*III).

employed a "Wound healing-like assay" that documents both adhesion of cells to gelatin and their migration. Thus, we could evaluate the effect of the ribozyme on both these processes. Figure 4 shows that the ribozyme specifically inhibits migration of the cells. The maximum inhibition of migration could be observed after 36 h indicating that this construct can be potentially useful as a gene-inactivating agent towards endothelial cells and may ultimately provide a therapeutic means to inhibit angiogenesis *in vivo.*

In conclusion, the presented data further support the role of β1 integrin receptors in endothelial cell migration, one of the early stages of angiogenesis. In addition to synthetic antisense oligonucleotides, DNAzymes and viral-mediated infection of endothelial cells to express antisense RNA, ribozymes may be useful inhibitors of the early stages of angiogenesis, particularly when specific blocking of the targeted integrin is concerned.

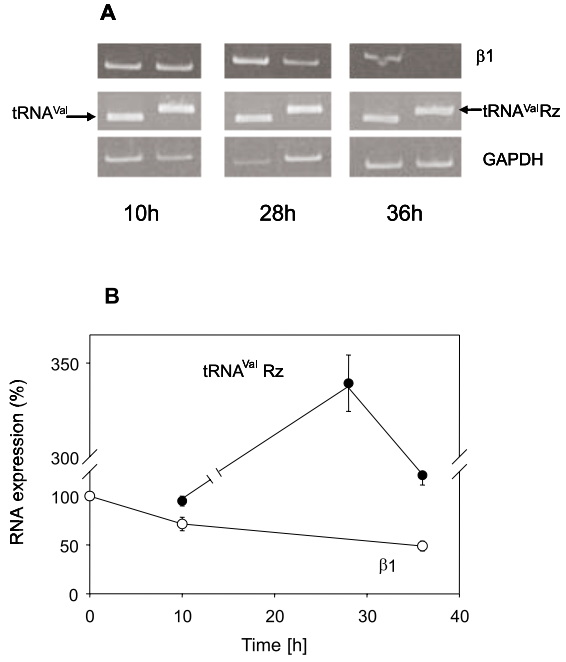

Figure 2. Time-course of intracellular concentration of the ribozyme mRNA and of the β1 integrin subunit mRNA.
Expression of β1 mRNA integrin subunit was analyzed 10, 28, and 36 h after EA.hy 926 transfection with pUC-KE-tRNA-CTE-Rz ($tRNA^{Val}$ Rz) or pUC-KE-tRNA-CTE ($tRNA^{Val}$) (negative control). In the same period of time, intracellular concentration of ribozyme mRNA was analyzed by RT-PCR. Panels A and B show gels after transfection of cells with 4 μg of plasmids and expression of the analyzed RNAs quantitated after densitometric scanning, respectively.

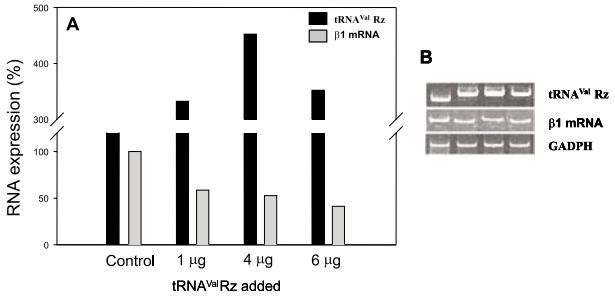

Figure 3. Intracellular concentration of the ribozyme mRNA and β1 integrin subunit mRNA after transfection of endothelial cells.
Both RNAs were evaluated by RT-PCR, 28 h after transfection of EA.hy 926 with different concentrations of pUC-KE-tRNA-CTE-Rz ($tRNA^{Val}$Rz). pUC-KE-tRNA-CTE served as a negative control for analysis of the level of β1 integrin subunit mRNA. Panels A and B show expression of the analyzed RNAs quantitated after densitometric scanning of gels and original gels showing the intensity of ribozyme mRNA, β1 integrin subunit mRNA, and GADPH mRNA respectively.

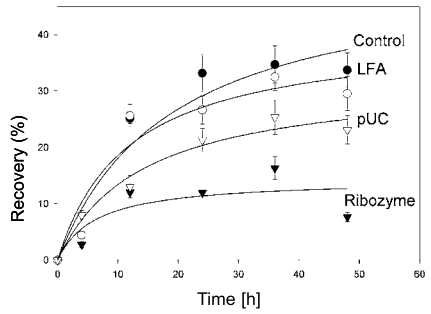

Figure 4. Inhibition of EA.hy 926 migration by ribozyme to β1 integrin subunit mRNA.
The effect of ribozyme was tested in a Wound healing-like assay and compared with control experiments, in which migration was analyzed in the absence of ribozyme, in the presence of lipofectamine or empty pUC vector.

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