DNAzymes to $\beta_1$ and $\beta_3$ mRNA Down-regulate Expression of the Targeted Integrins and Inhibit Endothelial Cell Capillary Tube Formation in Fibrin and Matrigel*

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A novel approach based on DNA-cleaving deoxyribozymes (DNAzymes) was developed to control expression of $\beta_1$ and $\beta_3$ integrins in endothelial cells. To engineer a specific cleavage site in mRNA, the flanking domains of DNAzymes were derived from oligodeoxynucleotides complementary to sequences corresponding to 1053–1070 and 1243–1267 in $\beta_1$ and $\beta_3$ mRNA, respectively. Phosphorothioate analogues of these antisense oligodeoxynucleotides, designated $\beta_1$-1053 and $\beta_3$-1243, significantly inhibited expression of $\beta_1$ and $\beta_3$ integrin subunits in endothelial and K562 cells at the level of mRNA and protein synthesis. They also significantly decreased the cell surface expression of corresponding subunits in endothelial cells and K562 cells, as measured by flow cytometry. In functional tests, $\beta_1$-1053 and $\beta_3$-1243 markedly reduced adhesion of cells to fibronectin and vitronectin, respectively. We designed DNAzymes to $\beta_1$ and $\beta_3$ mRNAs containing a 15-deoxynucleotide catalytic domain that was flanked by two substrate recognition segments of 8 and 10 deoxynucleotides for $\beta_1$ and $\beta_3$ DNAzymes, respectively. Both DNAzymes in the presence of Mg$^{2+}$ specifically cleaved their substrates, synthetic $\beta_1$ and $\beta_3$ mRNA fragments. Although DNAzymes were partially modified with phosphorothioate and with 2'-O-methyl groups at both the 5' and 3' ends indicated similar kinetic parameters, they were significantly more potent than the unmodified DNAzymes because of their much higher resistance to nuclease degradation. Similar to the antisense oligonucleotides, DNAzymes abolished microvascular endothelial cell capillary tube formation in fibrin and Matrigel. In conclusion, DNAzymes to $\beta_1$ and $\beta_3$ mRNAs with 2'-O-methyl modifications are potentially useful as gene-inactivating agents and may ultimately provide a therapeutic means to inhibit angiogenesis in vivo.

Angiogenesis is a multistep sequence of cellular reactions beginning with degradation of extracellular matrix and then proliferation and migration of endothelial cells followed by lumen formation and maturation (1). The formation of new blood vessels is critical to the development of normal tissues as well as the growth of solid tumors and to a large extent depends on specific molecular interactions between vascular cells and extracellular matrix (2, 3). Aberrant angiogenesis is also a key process for the growth of many disorders including atherosclerosis (4), diabetic retinopathy (5) and restenosis (6). Currently, two groups of integrin receptors are known to regulate adhesive interactions during angiogenesis. Integrins in the $\beta_1$ subfamily, particularly $\alpha_\beta_1$, and $\alpha_\beta_1$, which are substantially expressed in human angiogenic blood vessels, promote endothelial cell morphogenesis, migration, and tube formation (7, 8). The second group is composed of vitronectin receptors $\alpha_\beta_3$ and $\alpha_\beta_5$, which are poorly expressed in quiescent vasculature (9, 10) but can be substantially expressed by neoplastic vasculature and cells (11, 12). Expression of $\alpha_\beta_3$ and $\alpha_\beta_5$ can be differentially up-regulated by angiogenic inducers such as vascular endothelial growth factor and basic fibroblast growth factor (13). Vascular endothelial growth factor can also activate both $\beta_1$ receptors, and thus it enhances cell adhesion and migration (14). Furthermore, integrin-mediated adhesion leads to intracellular signaling events that regulate cell survival, proliferation and migration (7).

Given the importance of cell-cell and cell-matrix interactions in the pathogenesis of many human diseases, integrin receptors become very attractive targets for therapeutic intervention. Both peptide antagonists and the anti $\alpha_\beta_3$ monoclonal antibody LM609 (15) are potent angiogenesis blockers in animals and humans (16, 17). The therapeutic potential of $\alpha_\gamma$ integrin antagonists has been evaluated in phase I and phase II clinical trials for patients with late stage cancer (7). Regulation of integrin expression at the level of protein synthesis, either by synthetic oligodeoxynucleotides (16, 17) or viral-mediated infection of endothelial cells to express the antisense RNA endogenously (18), has been suggested as an alternative method to inhibit angiogenesis in vivo.

Recently DNAzymes were described (19) as conceptually similar to conventional hammerhead ribozymes able to specifically cleave RNA molecules with any sequence. These DNAzymes consist of a 15-deoxyribonucleotide catalytic domain flanked by two substrate recognition domains of seven to nine deoxyribonucleotides each, which bind the RNA substrate through complementary base-pairing. DNAzymes were found

*This work was supported by projects Z-KBN 004/PO4/98 from the Polish Committee for Scientific Research and CRP/Pol 98-02 from International Centre for Genetic Engineering Biotechnology (ICGEB) in Triest, Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: DNAzyme, DNA-cleaving deoxyribozyme; HUVEC, human umbilical vein endothelial cells; DMEM, Dulbecco’s modified Eagle’s medium; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; MeO-β1DE, 2'-O-methyl β1 DNAzyme.
to be potentially useful as gene-inactivating agents in the treatment of diseases such as chronic myelogenous leukemia (20). In contrast to conventional antisense oligodeoxynucleotides, they were highly specific and cleaved only aberrant BCR-ABL (fusion gene for p210BCR-ABL, a marker of chronic myelogenous leukemia) mRNA. In this report, we present data showing that DNAzymes to β1 and β3 mRNA reduce expression of targeted integrin subunits and block proliferation, migration, and network formation in a fibrin and Matrigel matrix.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Oligodeoxynucleotides Antisense to β1 and β3 mRNAs—**Oligodeoxynucleotides antisense to various regions of mRNA for both β-integrin subunits (Table I) were prepared on solid support using an ABI-394 DNA synthesizer as described previously (21). The most inhibitory oligodeoxynucleotides, β1-1053 (CAAGTGTGACAAATA-GAAC) and β3-1243 (TTGATGCCACATAGAGTGGCC) were used to construct type II DNAzymes. The first one, designated βDE, was obtained after insertion of a 15-deoxyribonuclease catalytic domain, ggttagctacaag, between sequences CAAGTGTG and AAGATGAAC, used as the flanking regions. In the second DNAzyme, β3DE, sequences GAGTCCCTA and AAGACTTGG were the antisense arms that recognized the target sequence in β3 mRNA. In addition, two inactive DNAzymes, β1DEin and β3DEin with structure designed based on β1-358 and β3-1070, were synthesized and used in control experiments. Both, β1-358 and β3-1070 did not show any effect on expression of β1 and β3 subunits in endothelial cells, respectively. The purity of antisense oligodeoxynucleotides and DNAzymes was confirmed by polyacrylamide gel electrophoresis.

**Preparation of Target RNA Substrates and Kinetic Analysis—**Aliquots of RNA substrates (20 μl; 5 μM) dissolved in a T4 polynucleotide kinase buffer were mixed with [γ-32P]ATP (2 μl; 20 μCi) and T4 polynucleotide kinase (3 units). Reaction was carried out for 1 h at 37 °C. All reported kinetic values were determined in multiple turnover reactions. Vmax and Km values were determined from the y-intercept and slope, respectively, of the best-fit line to a Linewaver-Burk plot of 1/V versus 1/[S]. Reactions (10 min, 37 °C, total volume 20 μl) were carried out in 50 mM Tris, pH 8.0, containing 15 mM MgCl2, 0.01% SDS, DNAzyme (0.0125 μM β3DNAzyme or 0.025 μM β1DNAzyme), and radiolabeled RNA substrate used in a wide range of concentrations. The cleavage reaction was stopped by the addition of 5 μl of 0.5 X EDTA, and products were separated by electrophoresis in 20% polyacrylamide gels under denaturing conditions. The Amount of product was evaluated by autoradiography of the excised gels.

**Cell Cultures—**Human umbilical vein endothelial cells (HUVEC) were isolated from freshly collected umbilical cords by collagenase treatment (22, 23). Cells were grown in gelatin-coated 75-cm² tissue culture flasks and were maintained at confluence in RPMI 1640 medium supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), fungizone (2.5 mg/ml), heparin (90 μg/ml), l-glutamine (1 mM), sodium bicarbonate (2 mg/ml), 20% fetal calf serum, and epidermal growth factor (40 ng/ml) at 37 °C in a humidified 5% CO2 atmosphere. Primary cultures were harvested at confluence with trypsin/EDTA and transferred into gelatin-coated dishes. In experiments, confluent cultures were used at the second passage. Human umbilical cell line EA.hy 926, derived by fusion of HUVECs with continuous human lung carcinoma cell line A549 were grown as monolayers in 25-cm² tissue culture flasks under standard conditions in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with HAT medium (hypoxanthine/aminopterin/thymidine), streptomycin (100 μg/ml), penicillin (100 units/ml), and 10% fetal bovine serum at 37 °C in a humidified 5% CO2 atmosphere (24, 25). For the experiments, cells were transferred to 6-well dishes and used at 70% confluence in the DMEM supplemented with the components mentioned above. Antisense oligodeoxynucleotides were used at the final concentration of 0–5 μM. DNAzymes were mixed with LipofectAMINE suspended in Opti-MEM reduced medium (5 μg/ml) and diluted with DMEM to obtain the final concentration of 0.3 μM. After incubation for 24 h, cells were detached with 1 ml EDTA (5 min at room temperature) and used for other experiments. The K562 cell line was routinely maintained in suspension in RPMI 1640 (Invitrogen) with 10% fetal bovine serum at 37 °C in a humidified 5% CO2 atmosphere. The cells were continuously kept at their logarithmic growth phase by diluting and supplying them with fresh culture medium every 2 or 3 days. All cultures were free of mycoplasma. Only cell cultures having less than 1% dead cells were used. Usually, samples were denatured at 95 °C for 5 min and then cooled down on ice. After that step, enzyme was added, and 25–30 primer extension cycles consisting of a 30-s denaturation step at 94 °C, a 30-s annealing step at 56 °C (61 °C for β3 mRNA), and a 40-s polymerase extension step at 72 °C were performed. Finally, each reaction was terminated with a 10-min elongation step at 72 °C. To detect glyceroldehyde-3-phosphate dehydrogenase (GAPDH), the same protocol was sized according to the manufacturer's instruction using (dT)20 primer.

**Relative Quantitative Reverse Transcription-PCR Assays of mRNA—**Total mRNA was purified from 1 × 106 cells using a Total RNA Prep Plus Kit (A&K Biotechnology). The first-strand cDNA was synthesized according to the manufacturer’s instruction using (dT)20 primer. Then, mRNAs of β1 and β3 integrin subunits were determined by relative quantitative RT-PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. The β1 mRNA assay employed the following oligodeoxynucleotide primers: 5'-GGTGCAATGAAGGGCGTGTTGG-3' and 5'-GGGAAATGGTGTTTG-CAAGTGTGaag-3'. To amplify β3 mRNA, primers 5'-CCT TTT CTG ATT GTG ACT GTG TTG-3' and 5'-GGC GAC ACC AGA TAG ACC ACA GAG G-3' were used. Usually, samples were denatured at 95 °C for 5 min and then cooled down on ice. After that step, enzyme was added, and 25–30 primer extension cycles consisting of a 30-s denaturation step at 94 °C, a 30-s annealing step at 56 °C (61 °C for β3 mRNA), and a 40-s polymerase extension step at 72 °C were performed. Finally, each reaction was terminated with a 10-min elongation step at 72 °C. To detect glyceroldehyde-3-phosphate dehydrogenase (GAPDH), the same protocol was used with oligodeoxynucleotide primers 5'-GAGAGATGATGAC-CCCTTTGAG-3' and 5'ACGAGATCCAGCAGG-3'.
K562 cells were incubated with 2.0 antisense to their mRNA. 

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Control [50x105]p3 mRNA

GAPDH mRNA

Panel A shows the relative expression of p3, β1 (260 bp), β3 (235 bp), and GAPDH (154 bp) mRNAs, were separated by electrophoresis in 7% polyacrylamide gels in TAE buffer (0.04 M Tris, 0.02 M CH₃COOH, 0.001 M EDTA) using the genetic size marker 100-bp DNA Ladder (Promega). Bands were visualized by UV light, and the results were recorded photographically and analyzed densitometrically. The concentrations of mRNA for β3 and β1 were normalized in each sample in relation to GAPDH mRNA.

Detection of Integrin Subunits by Western Immunoblotting—The protein content of lysates was determined by the BCA method (26). Cell lysate aliquots containing 20 μg of protein were boiled with 4× concentrated sample buffer, separated by SDS-PAGE (27), and then transferred electrophoretically to nitrocellulose membrane (0.45-μm pore size). β3 and β1 integrin subunits were detected with monoclonal antibodies. Immunodetection was accomplished using the enhanced chemiluminescence kit (ECL kit, Amersham Biosciences, Inc.), and then films were scanned and protein bands quantitated using the GellImage system (Amersham Biosciences, Inc.). To quantify the densitometric scans, the background was subtracted, and the area for each protein peak was determined.

Flow Cytometry—Subconfluent EA.hy 926 cells were harvested and washed with serum-free DMEM. Cells (1 × 10⁵) suspended in DMEM containing 1% bovine serum albumin were incubated in the dark at 4°C for 30 min with fluorescein isothiocyanate-conjugated monoclonal antibodies against β3 or β1 subunits (Dako). After double washing with 1% bovine serum albumin/phosphate-buffered saline, the cells were fixed by mixing the sedimented cells with 1% paraformaldehyde in phosphate-buffered saline and resuspended in appropriate fluid for flow cytometry analysis. Cell fluorescence was measured with a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA). The data were analyzed with PC Lysis II software.

Adhesion Assay—Wells of PS Maxisorp loose Nunc-Immuno™ modules (Nunc™ brand products) were coated with 50 μl of fibronectin at 10 μg/ml in TBS (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.6). Proteins were allowed to bind for 2 h at 37°C before the wells were rinsed twice with TBS and blocked for 1.5 h at 37°C in a humidified 5% CO₂ atmosphere with 200 μl of 1% heat-denatured bovine serum albumin in TBS, pH 7.5, containing 0.1 mM CaCl₂. Cells were harvested and resuspended in TBS containing 0.1 mM CaCl₂, pH 7.5. The total cell-associated protein was determined by dissolving the attached cells directly in the microtiter wells with 200 μl of Pierce BCA protein assay reagent (Pierce). The modules were incubated for 30 min at 37°C, and the absorbance of each well was determined at 562 nm with a microplate reader (BioKinetics Reader EL340, Bio-Tek Instruments).

Capillary Tube Formation by EA.hy 926 Cells Sandwiched between Fibrin II Gels (28)—Fibrinogen (1.5 mg/ml) was dialyzed into 0.05 M Tris, 0.15 M NaCl and passed through collagen immobilized on Sepharose 4B to remove fibronectin. To make the underlying fibrin gel, 250 μl of fibrinogen solution was placed into each well of a 24-well culture plate, and human thrombin (100 units/ml) was added to a final concentration of 2 units of thrombin/mg of fibrinogen. After the gels were polymerized (at least 5 min at 37°C), 1-ml aliquots of EA.hy926 cells (5 × 10⁵ cells/ml), suspended in serum-free endothelial cell basal medium supplemented as indicated above, were seeded onto each well. After 24 h, unattached cells were aspirated, and the same procedure was used to generate a second fibrin II gel overlaying the apical surface of the cells. This fibrin II gel was allowed to polymerize for 5 min at 37°C, and then 1-ml aliquots of fresh, supplemented, serum-free endothelial cell basal medium were added to each well. Tube formation was assessed at several different focal planes through the gel. The extent of capillary tube formation was judged in relation to the amount of endothelial cell monolayer and to the number, width, and length of the tubes formed. Re-embedding procedure was performed according to Nehls et al. (29). Briefly, the primary fibrin gels cultivated with EA.hy 926 cells were dissected into small pieces using scalpel blades. The pieces were then suspended in fibrin solution. After polymerization, over the next 3 days, the cells covering the fibrin fragments migrated into the surrounding second gel. As noted previously, under these conditions cellular

FIG. 1. Expression of β3 and β1 integrin subunits in K562 cells treated with different phosphorothioate oligodeoxynucleotides antisense to their mRNA. K562 cells were incubated with 2.0 μM oligodeoxynucleotide for 48 h, and then expression of β3 mRNA was evaluated by relative quantitative RT-PCR (A). Samples of the same cells were extracted with the SDS sample buffer and separated by SDS-PAGE followed by Western immunoblotting (B). Panel C shows relative expression of β3 at the level of mRNA and protein. RNA bands were visualized by UV light, recorded photographically, and analyzed densitometrically. The concentrations of mRNA for β3 were normalized in each sample in relation to GAPDH mRNA. Immunodetection of β3 was accomplished using an enhanced chemiluminescence kit, and then densitometric scans, the background was subtracted, and the area for each protein peak was determined. Panel D shows the relative expression of β1 in K562 cells after treatment with oligodeoxynucleotides antisense to β1 mRNA evaluated by the same approach described for β3 subunit.
Capillary Tube Formation in Matrigel—Wells of a 24-well plate were coated with Matrigel according to the manufacturer’s instructions (Becton Dickinson) and incubated at 37 °C for 30 min. HUVECs or EA.hy 926 cells were grown in 6-well dishes in the presence of DNAzymes (0.3 μM) mixed with LipofectAMINE (5 μg/ml) for 24 h. Then they were detached with 1 mM EDTA, sedimented by centrifugation for 5 min, and resuspended in cell culture medium. HUVECs or EA.hy 926 cells were added to Matrigel-coated wells and incubated for 24 h. Photomicrographs at 100× magnification were taken using a digital camera Photoclin AGFA attached to a Nikon TMS-F microscope.

Data Analysis—All values are expressed as the mean ± S.D. and were compared with controls and among separate experiments. Spearman rank correlation coefficients and Pearson’s product moment statistical tests were done. Because nonparametric statistical tests yielded very similar results to parametric tests, the latter are presented. Significant difference was taken for p values less than 0.05.
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RESULTS

Selection of the Flanking Sequences of DNAzymes—The target site for the specific cleavage of $\beta_1$ or $\beta_3$ mRNA is determined by sequences of the flanking deoxyribonucleotides that bind the RNA substrate via Watson-Crick base-pairing. Therefore, series of unmodified phosphodiester oligodeoxynucleotides and their phosphorothioate analogues were synthesized and tested (Table I). The sequences selected for antisense experiments were designed to avoid regions of high homology among $\beta$-subunits (30). In screening tests, the effect of phosphorothioate oligodeoxynucleotides on expression of $\beta_1$ and $\beta_3$ integrin subunits was analyzed using endothelial cell line EA.hy 926 and K562 cells. The cells were treated with oligodeoxynucleotides at the concentration of 2.0 $\mu$M, and the expression of integrin subunits was evaluated at the level of mRNA and protein synthesis by semiquantitative relative RT-PCR and Western immunoblotting, respectively. Fig. 1 shows that one of six oligodeoxynucleotides antisense to $\beta_1$ mRNA inhibited expression of $\beta_1$ subunit in K562 cells, both at the level of mRNA (panel A) and of protein synthesis (panel B). In similar screening analyses of 10 oligodeoxynucleotides antisense to the $\beta_3$ integrin subunit, only one, $\beta_1$-1053, showed inhibitory activity and significantly reduced $\beta_1$ expression. When compared with untreated cells, expression of $\beta_3$ subunit in K562 cells incubated with oligonucleotide $\beta_3$-1243 was reduced in 80 and 50% as measured at the level of mRNA and total protein, respectively (Fig. 1C). Treatment of the cells with $\beta_1$-1053 resulted in inhibition of $\beta_1$ expression in 45 to 55% (Fig. 1D). The specificity and efficacy of inhibition produced by $\beta_3$-1243 and $\beta_1$-1053 were further tested and compared with that obtained with three phosphorothioate congeners possessing scrambled sequence, sense sequence, and sequence containing two mismatches. The inhibitory effects produced by both antisense oligonucleotides were concentration-dependent and -specific as evidenced in Fig. 2. The total concentration of $\beta_1$ or $\beta_3$ integrin subunit was reduced in 61% (Fig. 2A) and 50% (Fig. 2C) at 2.0 $\mu$M $\beta_1$-1053 or 2.0 $\mu$M $\beta_3$-1243 oligodeoxynucleotide, respectively. Treatment of EA.hy 926 cells with control oligonucleotides, including that which was only slightly modified by introducing two mismatches in the central region of $\beta_1$-1053 (CAG GTG CAA TTT AAG), showed only a minor effect on expression of $\beta_1$ mRNA (Fig. 2B). Similarly, incubation of K562 cells with 2 $\mu$M $\beta_3$-1243 resulted in significant reduction of $\beta_3$ mRNA concentration (Fig. 2D).

We next asked whether treatment of cells with antisense oligonucleotides also affects the surface exposed $\beta_1$ and $\beta_3$ integrins. In addition to endothelial cell line EA.hy 926, in these experiments we used pluripotent K562 cells that upon activation with phorbol ester differentiate along the megakaryocytic pathway. Consistent with previous observations (31), differentiation of K562 cells induced by PMA was associated with a significant increase in the expression of several receptors belonging to the $\beta_1$ and $\beta_3$ integrin subfamilies (Fig. 3). Treatment of K562 cells with $\beta_1$-1053 oligonucleotide for 48 h reduced the surface expressed $\beta_1$ integrin subunit in 35% (Fig. 3B). Oligodeoxynucleotides antisense to other regions of $\beta_1$ and $\beta_3$ mRNA, which did not reduce expression of either
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mRNA or the total pool of those integrin subunits, were used as controls. The same effect was observed when the endothelial cell line EA.hy 926 were treated with antisense oligodeoxynucleotides, then detached with EDTA, and after labeling with either anti-β1 or anti-β3 antibodies conjugated with fluorescein, subjected to flow cytometry. Under these conditions, β3-1243 oligonucleotide produced a 30% inhibition of β3 integrin subunit expression in endothelial cells (Fig. 3C).

Treatment of K562 cells and EA.hy 926 cells with oligonucleotides antisense to β3 mRNA, particularly by β1-1053 and β3-1243, resulted in a significant inhibition of adhesion to fibronectin and vitronectin, respectively (Fig. 4). In this assay, cells were first preincubated with 2.5–5.0 μM oligonucleotides for 24–72 h. Then their adhesion to either fibronectin or vitronectin was analyzed for 1.5 h at 37 °C. The extent of adhesion was evaluated based on the amounts of cellular protein detected as associated with plastic wells. The most inhibitory oligonucleotides, β1-1053 and β3-1243, inhibited adhesion by 40–45%.

Design of DNAzymes and Cleavage Activities—Based on the aforementioned analysis, two DNAzymes were constructed. The first one, 5′1DE, contained fragments of β1-1053 oligodeoxynucleotide, CAAGGGTGAAG and AATAGAAG, as the antisense arms that recognize the target sequence in β3 mRNA. In the second DNAzyme, 5′3DE, sequences of β3-1243, namely GAGTCCGATA and AAGACTTGGG, were used as the flanking regions. In both cases, a 15-deoxynucleotide catalytic domain, getagctcatacg, originated from a type II DNAzyme was incorporated between the flanking arms (Fig. 5A). The efficiency of catalysis in vivo depends upon specific recognition of the target sequence and resistance of DNAzymes to nucleases. To increase stability of DNAzymes in vivo, modified oligonucleotides such as phosphorothioate analogues and 2′-O-methyl-substituted residues were introduced at both the 5′ and 3′ sides. Hence, S-β1DE and S-β3DE have two phosphorothioate substitutions, whereas MeO-β1DE and MeO-β3DE contain two 2′-O-methyl-substituted residues at both their 5′ and 3′ sides.

To study the nuclease resistance of the modified DNAzymes, their stability was analyzed in human serum. 32P-Labeled oligonucleotides were incubated in 90% human serum up to 24 h at 37 °C and the products were analyzed at different time points by electrophoresis on a 20% polyacrylamide gel. As seen in Fig. 5B, 5′1DE and 5′3DE were degraded more rapidly than their modified analogues, which remained intact in substantial amounts even after 6 h of incubation. This finding indicates that introducing two phosphorothioate linkages to two 2′-O-methyl-substituted residues at both the 5′ and 3′ ends significantly enhanced their resistance to nucleases present in blood serum.

Cleavage Activities and Specificities of DNAzymes to β1 and β3 mRNA—Before transfecting endothelial cells with DNAzymes, their effects on synthetic substrates were evaluated using 18- and 21-mer nucleotides, corresponding to fragments of β1 and β3 mRNA (Fig. 5A). The relative cleavage activities and the specificities of each DNAzyme for the substrates labeled with 32P were shown in Fig. 6. Reactions were performed in 50 mM Tris-HCl, pH 8.0, containing 15 mM MgCl2, under enzyme-saturating conditions. 32P-Labeled m6A RNA substrates were mixed with 0.025 μM β1 DNAzymes (5′1DE, S-β1DE, MeO-β1DE) or 0.0125 μM β3 DNAzymes (5′3DE, S-β3DE, MeO-β3DE) in the molar ratio ranging from 5:1 to 80:1 and incubated at 37 °C, and aliquots were withdrawn at different time points. The cleavage reaction was stopped by addition of 5 μl of 0.5 M EDTA, and products were separated by electrophoresis in 20% polyacrylamide gels under denaturing conditions. The amount of product was evaluated using a PhosphoImager. Fig. 6A shows a composition of cleavage mixtures obtained after a 60-min incubation of 32P-labeled mRNA substrates with 0.025 μM DNAzymes added to the molar ratio ranging from 5:1 to 80:1. Each of the DNAzymes, unmodified (5′1DE and 5′3DE) and modified (MeO-5′1DE and MeO-5′3DE), cleaved the substrate at the predicted site. As observed previously (20), neither the thio substitutions nor the 2′-O-Me resi-
differences inhibited the cleavage activity. DNAzymes to both \( \beta_1 \) and \( \beta_3 \) mRNA were highly specific, and they did not cleave the nonspecific substrate, for example \( \beta_3 \) and \( \beta_1 \) mRNA substrates, respectively (not shown).

To further characterize enzymatic properties of the DNAzymes, kinetic parameters for the cleavage of synthetic substrates were determined (Fig. 6, B and C) and are summarized in Table II. In these experiments cleavage of the synthetic products was evaluated by scanning autoradiograms obtained from exposure of gels to Kodak film. Comparisons of kinetic parameters revealed significant differences in \( V_{max} \), \( K_m \), and \( k_{cat} \) values between the modified and unmodified DNAzymes. For example, introducing two 2’-O-methyl-substituted residues at both the 5’ and 3’ sides of DNA enzymes to \( \beta_1 \) and \( \beta_3 \) subunits resulted in opposite changes of \( K_m \) values when compared with unmodified DNAzymes (Table II). Both methylated DNAzymes showed higher \( V_{max} \) and \( k_{cat} \) values, but in terms of \( k_{cat}/K_m \), only MeO-\( \beta_3 \)DE appeared to be more powerful than the unmodified DNAzyme toward synthetic mRNA substrates.

Inhibition of Microvascular Endothelial Cell Capillary Tube Formation in a Fibrin and Matrigel Matrix—To identify DNAzymes to \( \beta_1 \) and \( \beta_3 \) integrin subunits as potential angiogenesis modulators, their effects were tested in three-dimensional fibrin and Matrigel gel. In these experiments, cleavage of the synthetic mRNA were highly specific, and they did not cleave the nonspecific substrate, for example \( \beta_3 \) and \( \beta_1 \) mRNA substrates, respectively (not shown).

All reported kinetic values were determined in multiple turnover reactions. \( V_{max} \) and \( K_m \) values were determined from the Lineweaver-Burk plot of \( 1/V \) versus \( 1/[S] \) intercept and slope, respectively, of the best-fit line to a Lineweaver-Burk plot of \( 1/V \) versus \( 1/[S] \).

| Integrin subunit | DNAzyme | \( V_{max} \) (\( \mu \)M/min) | \( K_m \) (nM) | \( k_{cat} \) (1/min) | \( k_{cat}/K_m \) (10^6) |
|-----------------|---------|-----------------|---------|-----------------|-----------------|
| \( \beta_1 \)   | \( \beta_{1\text{DE}} \) | 0.0227 ± 0.0015 | 0.1354 ± 0.0056 | 0.9094 ± 0.0594 | 6.73 × 10^6 |
|                 | MeO-\( \beta_{1\text{DE}} \) | 0.0391 ± 0.0113 | 0.0303 ± 0.1334 | 1.5623 ± 0.4526 | 5.34 × 10^6 |
| \( \beta_3 \)   | \( \beta_{3\text{DE}} \) | 0.0090 ± 0.0003 | 0.2981 ± 0.0439 | 0.3590 ± 0.0105 | 1.22 × 10^6 |
|                 | MeO-\( \beta_{3\text{DE}} \) | 0.0210 ± 0.0013 | 0.827 ± 0.240 | 0.848 ± 0.099 | 1.08 × 10^6 |
|                 | S-\( \beta_{3\text{DE}} \) | 0.0223 ± 0.0043 | 0.492 ± 0.085 | 1.780 ± 0.345 | 3.62 × 10^6 |

\( \beta_1 \) and \( \beta_3 \) mRNA substrates.

![Control cells](http://www.jbc.org/)

![Control \( \beta_{3\text{DE}} \)in](http://www.jbc.org/)

![Control \( \beta_{1\text{DE}} \)in](http://www.jbc.org/)

![MeO-\( \beta_{3\text{DE}} \)](http://www.jbc.org/)

![MeO-\( \beta_{1\text{DE}} \)](http://www.jbc.org/)

**Fig. 7.** Inhibition of endothelial cell capillary tube formation in fibrin by DNAzymes to \( \beta_1 \) and \( \beta_3 \) mRNA. Endothelial cells 24 h after seeding into three-dimensional fibrin gel show irregular growth pattern and formation of numerous capillary-like structures. This process was not inhibited by active DNAzymes with the modified DNAzymes (Fig. 9A and B) and Matrigel. Upon treatment with active DNAzymes to \( \beta_1 \) and \( \beta_3 \), the cells are maintained as a monolayer and little invasion into the gel is seen. These cells showed a cobblestone-like growth or a rounded morphology. Representative fields from one of three experiments are shown at 150× magnification.

Inhibition of endothelial cell capillary tube formation in Matrigel was inhibited exclusively after reduction of \( \beta_3 \) subunit expression (Fig. 9). In these experiments the effect of DNAzymes to \( \beta_1 \) and \( \beta_3 \) was evaluated 24 and 72 h after plating of either HUVECs (Fig. 9A) or EA.hy 926 cells (Fig. 9B) on Matrigel. Under these conditions both primary and immortalized endothelial cells were induced to form a capillary network within the gel. Realignment of the endothelial cells, cell invasion into the gel, and the beginning of cell elongation was evident 6 h after plating the cells onto the gel, and capillary tube formation was clearly visible by 24 h, particularly in the case of HUVECs (Fig. 9A). Very little, if any, tube formation occurred after treatment of the cells with MeO-\( \beta_{1\text{DE}} \). Such cells were maintained as a monolayer on top of the gel, and little invasion into the gel was seen (Fig. 9, A and B). MeO-\( \beta_{3\text{DE}} \) and control inactive DNAzyme to \( \beta_1 \) had no effect.
on tube formation by both types of cells. This observation supports the specificity of this assay, indicating that expression of collagen-binding integrins belonging to the β1 subfamily is critical for the early stages of capillary tube formation in Matrigel. Although antisense oligodeoxynucleotides and DNA enzymes designed based on their structures did not inhibit completely the expression of targeted integrins, the reductions either in both β1 and β3 integrins or exclusively in β3 integrins appeared to be sufficient to substantially reverse the ability of endothelial cells to form capillary tubes in fibrin gels or Matrigel, respectively.

**DISCUSSION**

The adhesive interactions of cells with the extracellular matrix play an important role in the organization of cells in differentiated organs as well as in the motility, immune recognition, and aggregation of cells. These functions are mediated by a group of cell surface receptors known as integrins (36–39). The migration of vascular cells is also critical to the process of blood vessel remodeling. For instance, the migration of a subpopulation of endothelial cells is an important step in the multifactorial process of angiogenesis. Because integrin receptors are involved directly in the early stages of angiogenesis, they constitute a very attractive therapeutic target that could be either directly blocked or inhibited at the protein synthesis level. Different strategies aimed at rapid inactivation of integrin receptors have been developed, including neutralization of the target proteins by monoclonal antibodies and low molecular weight peptide analogues. Based on successful preclinical data, several anti-angiogenic agents directly reacting with integrins, used alone or in combination with conventional therapies, are now in clinical trials (40). However, there are several studies suggesting that certain integrin-specific ligands provoke trans-dominant inhibition that could lead to unexpected effects on cell migration, gene expression, and cell viability (41–45).

Alternatively, a reduction in the expression of these proteins has been reversibly accomplished by modifying their synthesis in the target cells. Synthesis of integrin receptors was successfully reduced by synthetic antisense oligonucleotides and endogenous antisense mRNA expressed upon transfection of endothelial cell lines. However, the regions of cDNA that yield effective antisense probes cannot be predicted with certainty because of the high homology among integrin subunits, particularly those belonging to the α and β subunits. On the other hand, the utility of such antisense probes is quite often questioned because of their low specificity (46). In contrast, DNAzymes were found to be very specific and cleaved mRNA precisely at the targeted sequence (20). Although DNAzymes are similar to conventional hammerhead ribozymes, their complexes with RNA are less stable than the corresponding RNA-RNA duplexes. Hence, mismatches in binding arms that might be generated during interaction with a nontarget mRNA, such as the mRNA of homologous integrin subunits, would interfere.
with cleavage activity to a greater extent than in the case of a ribozyme.

The catalytic domain used in this study was selected previously by combinatorial strategy (19) as a part of “10-23”deoxiribozyme. This catalytic core was ranked among the most effective nucleic enzymes of its kind, even when compared with natural self-cleaving ribozymes (47). The design of the DNAzymes and the choice of target sites for the specific cleavage of $\beta_1$ and $\beta_3$ mRNA were based on analysis of the accessibility of different mRNA sequences to antisense oligodeoxynucleotides. We found two such regions that corresponded to residues 1053–1070 and 1243–1267 of $\beta_1$ and $\beta_3$ mRNA, respectively. As demonstrated under “Results,” phosphorothioate analogues of oligodeoxynucleotides complementary to these sequences because of antisense effects specifically reduced expression of the relevant integrin subunit, measured at mRNA levels, protein synthesis, and on the surface of the cells. Thus, both $\beta_1$(1053–1070) and $\beta_3$(1243–1267) mRNA sequences appeared to be easily accessible to antisense probes and were used to design flanking regions of DNAzymes. Antisense oligonucleotides and DNAzymes to $\beta_1$ and $\beta_3$ integrin subunits were highly effective inhibitors of endothelial cell adhesion and capillary tube formation.

In conclusion, the data presented here further support the roles of $\beta_1$ and $\beta_3$ integrin receptors in endothelial cell migration and in the early stages of angiogenesis. In addition to synthetic antisense oligonucleotides and virally mediated infection of endothelial cells to express endogenously the antisense RNA, DNAzymes can be very useful inhibitors of angiogenesis, particularly when the specific blocking of the targeted integrin is concerned.

Acknowledgment—We thank Dr. M. Maszewska for excellent help in maintaining the endothelial cell cultures.

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DNAzymes to $\beta_1$ and $\beta_3$ mRNA Down-regulate Expression of the Targeted Integrins and Inhibit Endothelial Cell Capillary Tube Formation in Fibrin and Matrigel

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J. Biol. Chem. 2002, 277:6779-6787.
doi: 10.1074/jbc.M102325200 originally published online October 23, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102325200

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