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Cellular delivery of hammerhead ribozymes conjugated to a transferrin receptor antibody

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Abstract

Chemically-modified hammerhead ribozymes are sequence-specific RNA enzymes that can cleave target mRNA. These molecules have potential application as biological tools to understand gene expression and as therapeutic agents for the selective down-regulation of genes implicated in disease. However, as a result of their polyanionic character and relatively large molecular weights, ribozyme delivery to target cells is relatively inefficient. Using nuclease resistant 2′-*O*-methyl-modified ribozymes targeting the c-*erb*B1 oncogene, we have evaluated the potential use of human monoclonal transferrin-receptor antibody (TRA)-ribozyme conjugates for the improved delivery of ribozymes to A431 tumour cells. A 37-mer ribozyme derivatized with a free thiol-group at the 5′-end and bearing an internal [^{32}P]-radiolabel was conjugated to either TRA or a non-specific IgG antibody using the heterobifunctional crosslinker, succinimidyl 4-(maleimido methyl)cyclohexane-1-carboxylate (SMCC). Up to six molecules of the ribozyme could be conjugated to one molecule of antibody. Cellular uptake studies in cultured human epidermoid A431 carcinoma cells showed that approximately a three-fold increase in cellular association could be obtained with the TRA-ribozyme conjugate compared to the free ribozyme. Cellular association of the conjugate was temperature-dependent and was inhibited by competition with excess free transferrin receptor antibody implying that conjugate uptake was consistent with the transferrin receptor-mediated endocytosis pathway. Treatment of cells with monensin further enhanced TRA-ribozyme conjugate cell association indicating that ribozyme delivery of conjugates may be further improved by strategies that modulate vesicular trafficking in cells. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Hammerhead ribozyme; Transferrin receptor antibody; Monensin; Cell uptake; Receptor mediated delivery; Antibody conjugates

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1. Introduction

Ribozymes are ribonucleic acid molecules which possess enzymatic properties and can be engineered to perform *trans*-cleavage of RNA molecules in the absence of proteins (Cech and Bass, 1986; Haseloff and Gerlach, 1988; Bratty et al., 1993). Ribozymes have the potential to inhibit gene expression in a sequence specific manner via the cleavage of mRNA molecules and they are under investigation as potential therapies for human malignancies and other diseases (for reviews see Marschall et al., 1994; Akhtar and Rossi, 1996; Usman and Stinchcomb, 1996; Birikh et al., 1997). Several ribozyme motifs have been isolated to date (for review see Symons, 1992; Symons, 1994) but the hammerhead is the most widely studied because its small size makes it more amenable to mechanistic investigation and allows preparation via automated, solid-phase chemical synthesis (Rao and MacFarlane, 1995; Wincott et al., 1995; Usman et al., 1996). Unmodified RNA is rapidly degraded by ribonuclease enzymes present in biological systems and consequently most studies of ribozyme efficacy have, until recently, involved the endogenous delivery of ribozymes via expression vectors. However, recent chemical advances have allowed site-specific chemical modifications to be introduced into the structure of the hammerhead and these can confer nuclease resistance (Heidenreich et al., 1994; Beigelman et al., 1995; Heidenreich et al., 1996; Fell et al., 1997). This has resulted in a number of publications which have demonstrated both ex vivo and in vivo efficacy of exogenously delivered ribozymes (Lyngstadaas et al., 1995; Sioud, 1996; Flory et al., 1996; Jarvis et al., 1996; Wan et al., 1998). However, the cellular uptake of modified ribozymes following exogenous delivery is thought to occur via a process of adsorptive or fluid-phase endocytosis (Fell et al., 1997) which appears to be relatively inefficient. Thus, delivery strategies which can enhance uptake will be required if ribozymes are to be administered via exogenous routes for therapeutic purposes.

The transferrin receptor (for review see Friden, 1993) represents a potential carrier system by which ribozymes could be internalised by cells following exogenous delivery. The physiological role of the transferrin receptor is to provide an efficient mechanism for the cellular uptake of iron-transferrin complexes. This process involves the internalisation of the receptor into endosomal vesicles following the binding of an iron-transferrin complex. The iron dissociates from the transferrin molecule upon reaching the acidified environment of endosomes and the (‘apo-’) transferrin molecule remains bound to the transferrin receptor which is recycled to the cell surface via the Golgi complex (Friden, 1993). In previous studies, the transferrin receptor has been utilised to allow low molecular weight drugs and antisense oligonucleotides to be internalised following complexation with the transferrin ligand (Citro et al., 1992; Wagner, 1994) or conjugation to anti-transferrin receptor antibodies (Bickel et al., 1993; Boado, 1995; Walker et al., 1995). In this study, we have investigated the possibility of conjugating chemically-stabilised ribozymes to the human monoclonal transferrin receptor-antibody (RVS-10) in an attempt to improve cellular association ex vivo. In addition, the use of monensin, a carboxylic acid ionophore that interferes with vesicular trafficking, was employed in an attempt to further enhance cellular delivery of ribozyme-antibody conjugates.

2. Materials and methods

2.1. Synthesis and radiolabelling of ribozyme sequences

Chimaeric, partially modified hammerhead ribozymes used as model compounds in these studies were designed to cleave a 3′-untranslated region of the human c-*erb*B1 mRNA (Genbank accession code: X00588) at position 5401 (see Fig. 1 for ribozyme structure and chemical modifications). Chimaeric ribonucleotide sequences were synthesised on an automated DNA/RNA synthesiser (Model 392, Applied Biosystems, Warrington, UK) using 5′-*O*-(DMTr)-2′-*O*-(Fpmp) ribonucleoside phosphoramidites, 2′-*O*-methylated nucleoside phosphoramidites and standard deoxynucleoside phosphoramidites (all supplied by Cruachem, Glasgow, UK). Sequences were synthesised, purified and deprotected according to the method of Rao and MacFarlane (1995) and the relevant manufacturers protocols (Cruachem). Ribozymes to be used directly in cellular uptake studies were synthesised in two halves, with the junction 5′- to the GAAA sequence in loop II (see Fig. 1). The synthesis of the two halves allowed the ribozyme to be internally radiolabelled using T4 RNA Ligase which catalyses the ATP-dependant formation of a phosphodiester bond between a 5′-terminal phosphate (of a 'donor' nucleotide) and a 3′-terminal hydroxyl (of an 'acceptor' nucleotide) (Romaniuk and Uhlenbeck, 1983; Tessier et al., 1986). The 3′-half portion of the hammerhead ribozyme was used as the 'donor' molecule in the ligation reaction. A 5′-[^{32}P]-phosphate radiolabel was added to the 3′-half of the ribozyme using [^{32}P]-γ-dATP (DuPont NEN) and bacteriophage T4 polynucleotide kinase (Gibco BRL) in 100 mM Tris, pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 0.2 mM spermidine and 0.2 mM EDTA at 37°C for 1 h as described previously (Akhtar et al., 1991). The radio-labelled 3′-half of the ribozyme was purified using a Nensorb-20 column (DuPont NEN) and was then 'blocked' at the 3′-terminus with (unlabelled) ddCTP using terminal transferase (Boehringer Mannheim) in accordance with the manufacturers' protocol for 3′-end labelling of oligonucleotides. The replacement of the 3′-terminal hydroxyl group with an additional 2′,3′ di-deoxynucleotide was designed to prevent the sequence acting as an 'acceptor' in the subsequent ligation reaction and thus, prevented the formation of multimeric products. Both halves of the ribozyme were purified separately using Nensorb-20 Columns. The radiolabelled 3′-half was ligated to the 5′-half portion of the ribozyme using T4 RNA Ligase 180 μg/ml (Pharmacia Biotech, Sweden) in 50 mM HEPES (pH 8.3), 10 mM $MgCl_2$, 10 g/ml BSA, 10% v/v DMSO and 100 M ATP (all obtained from Sigma, Poole, UK) at room temperature for 8 h. A 5:1 concentration ratio of donor:acceptor was used in this reaction. Internally radiolabelled ribozyme was separated from unreacted sequences by 20% native PAGE. The excised band containing the ribozyme, as detected by autoradiography, was eluted in 0.1% diethyl pyrocarbon treated water and concentrated by vacuum centrifugation.

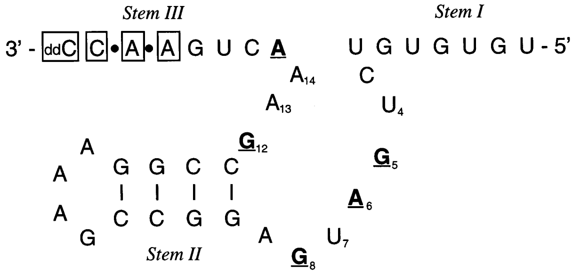

Fig. 1. Diagram showing the structure of the 37-mer chimaeric hammerhead ribozyme motif directed against the human c-*erb*B1 mRNA. (The numbering system of Hertel et al., 1992 has been used. Bases shown in bold and underlined represent unmodified ribonucleotides, boxed bases represent oligodeoxynucleotides (DNA bases). All other bases are 2′-*O*-methylated nucleotides. The symbol '●'denotes the position of phosphorothioate linkages. ddC represents the position of the 3′-terminal di-deoxy cytidine nucleotide).

Where the subsequent attachment of antibody molecules to the ribozyme was required, two different sections of the chemically modified ribozyme were synthesised and deprotected for use in the internal radiolabelling reaction. An 8-mer, fully 2′-*O*-methylated 'acceptor' sequence from the 5′-end of the ribozyme (5′-UGU GUG UC-3′) was synthesised and a 5′-end C_6 thiol modifier was attached during automated synthesis using C_6 thiol modifier cyanoethyl phosphoramidite monomers (0.1 M, Cruachem). The remainder of the hammerhead molecule (5′-U**GA** U**GA** GGC CGA AAG GCC **G**AA **A**CU *gaa*-3′, the 'donor' sequence, containing 2′-*O*-methylated nucleotides at all sites except for the bases shown in large bold type which were unmodified ribonucleotides and those in lower case which were oligodeoxynucleotides) was then synthesised and deprotected. This 'donor' sequence was 5′-end radiolabelled and 3′-blocked with ddCTP as described above. Following Nensorb-20 purification the 8-mer thiol-modified 'acceptor' sequence was ligated to the 29-mer 'donor' sequence with T4 RNA Ligase as described above. The resulting 37-mer internally radiolabelled hammerhead ribozyme bearing a C_6 thiol modifier group at the 5′-terminus (see Fig. 2A) was separated from unreacted sequences

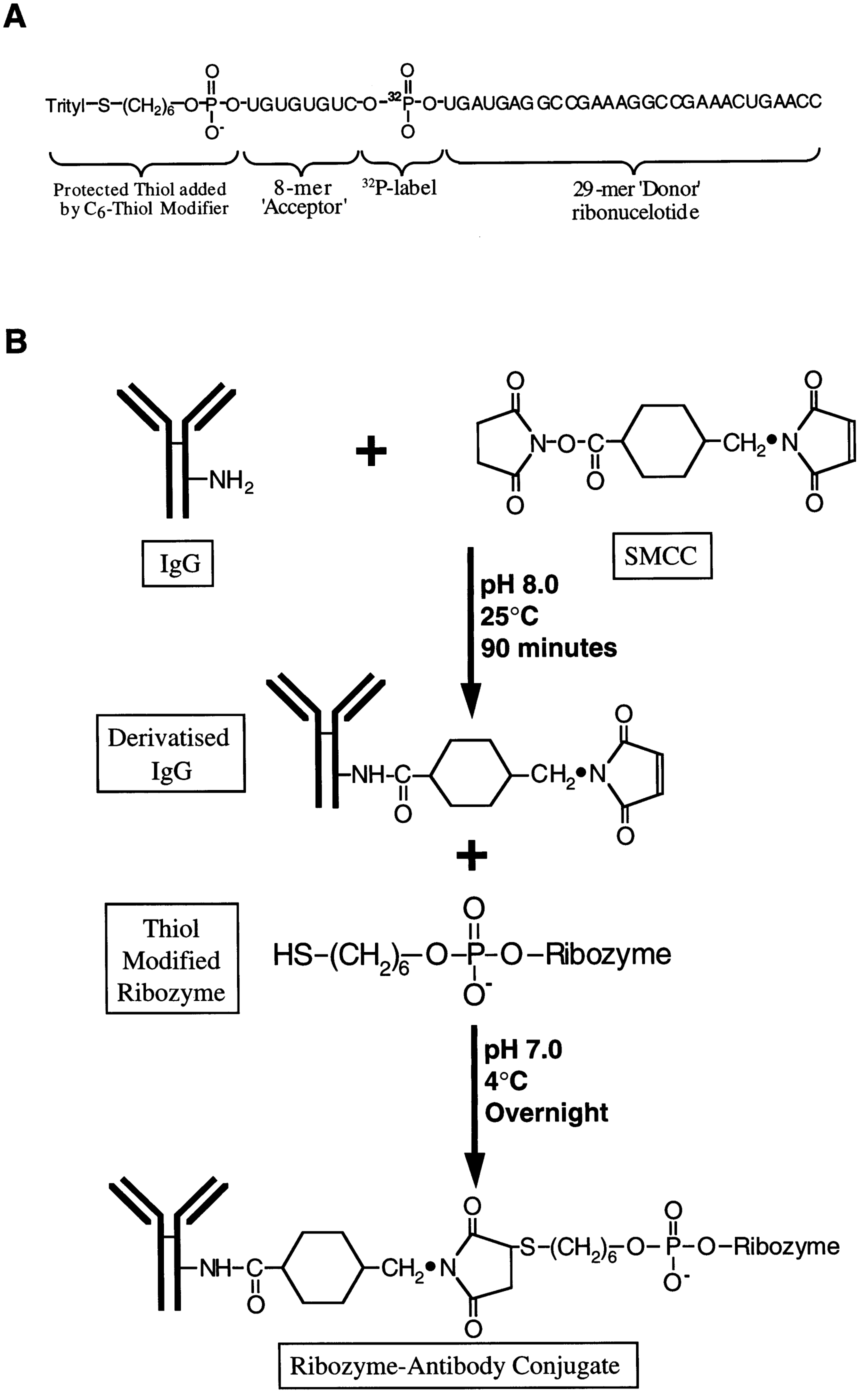

Fig. 2. A. Diagram showing the structure of the thiol-modified chimaeric hammerhead ribozyme used for the preparation of ribozyme-antibody conjugates. B. Schematic showing the synthesis of monoclonal antibody-ribozyme conjugates.

by 20% PAGE and was used to prepare ribozyme-antibody conjugates.

2.2. Derivatisation of monoclonal antibodies with the heterobifunctional crosslinker; SMCC

To a solution of mouse-anti-human transferrin receptor antibody (Clone RVS-10 (IgG1 subclass); Biogenesis, UK) or a control mouse-anti-human IgG (2–4 mg) (Sigma, Poole, UK) in 2.5ml of 50 mM phosphate buffer (pH 8.0) was added 0.05 M of the heterobifunctional crosslinker, succinimidyl 4-(maleimidomethyl) cyclohexane-1-carboxylate (SMCC) in dimethylformamide (DMF) solution and the derivitisation procedure followed as described previously (Walker et al., 1995).

2.3. Conjugation of thiol modified radiolabelled ribozyme to SMCC-modified monoclonal antibodies

5′-Thiol modified, internally [^{32}P]-radiolabelled, chimeric ribozymes were dissolved in 1 ml of the reaction buffer at pH 7.0 (50 mM phosphate buffer, 0.1 M NaCl and 5 mM EDTA) which had been thoroughly degassed with nitrogen and filtered using a 0.2 μm syringe filter (Sartorius, UK). Seven-hundred-and-fifty μg of the SMCC-derivatised monoclonal antibody (either transferrin receptor antibody or a control human IgG) was added to the ribozyme solution in 1 ml of the same reaction buffer and left overnight at 4°C. Unreacted maleimide groups introduced by the SMCC on the antibody were blocked with 0.01 M cysteamine for 2 h at room temperature. The conjugate was purified by gel filtration on Sephadex G-25 (1.6 × 20 cm column, Pharmacia Biotech) and eluted in 50 mM phosphate buffer (pH 7.4), containing 0.25 M NaCl which had been filtered using a 0.2 μm syringe filter (Sartorius, UK). The first 1–2 ml fraction which was eluted from the column contained the purified conjugate. The number of ribozyme molecules conjugated to the transferrin receptor antibody (TRA) was quantified spectrophotometrically by determination of the absorbance of the conjugate at 260 and at 280 nm. The degree of conjugation was then derived from the A260/A280 ratio (see Kuijpers et al., 1993). To confirm their constitution, the purified ribozyme-antibody conjugates were characterised in terms of their relative mobilities on 10% SDS–PAGE gels and in terms of their susceptibility to Proteinase K treatment (1.5 units/ml for 2 min at 37°C). The relative mobility of conjugate samples was also compared with that of unconjugated ribozyme and 'free' [^{32}P]-γ-dATP radiolabel (ICN Biomedicals, UK). Radioactive species were detected by autoradiography.

2.4. Cell culture and uptake studies

A431 cells were maintained at 37°C in a 5% CO_2 atmosphere in DMEM containing 10% foetal calf serum, 2 mM glutamine and with a final concentration in the medium of 1% v/v of penicillin and streptomycin (all from Gibco BRL). For uptake experiments, cells were seeded at 7.5×10^4 cells per well (24 well plates). Twenty-four h post-seeding, when the cells were approximately 70% confluent, cells were incubated with either internally radiolabelled ribozyme-antibody conjugates or internally radiolabelled ribozyme alone in serum free D-MEM medium containing 2 mM glutamine. For competition studies the non-derivatised anti-transferrin receptor antibody (TRA) was prepared at a 5-molar excess in serum-free media. Alternatively, a 100 μM solution (see Stein et al., 1984) of monensin (Sigma, UK [M-5273]) in serum-free media was prepared from a concentrated stock solution of monensin dissolved in Analar grade ethanol. Competing antibodies or monensin were pre-incubated with cells in ice-cold serum-free medium for 15 min, before re-warming to 37°C. Cellular association studies were then initiated using serum-free media, which was also supplemented with the relevant agent. After the desired incubation time (60 min to 4 h), the medium was removed and the A431 cells washed four times with 1 ml ice cold PBS. Cells were then lysed with 3% Triton X-100 and cell associated radioactivity was determined by liquid scintillation counting. The cell association studies were performed at either 4 or at 37°C.

3. Results and discussion

3.1. Synthesis and characterisation of ribozyme-antibody conjugates

This study aimed to evaluate TRA-ribozyme conjugates for the improved delivery of synthetic ribozymes to A431 epidermoid tumour cells. As unmodified RNA ribozymes are extremely unstable to nuclease digestion in the biological milieu, we synthesised 2′-*O*-methyl-modified chimaeric hammerhead ribozymes (see Fig. 1) as these are biologically stable with degradation half-lives in excess of 18 h in human serum (data not shown; see also Fell et al., 1997; Wan et al., 1998). Ribozyme-antibody conjugates were then synthesised as per the synthetic scheme outlined in Fig. 2B. To confirm the constitution of TRA-ribozyme conjugates (i.e. to confirm that the ribozyme was covalently linked to the antibody) conjugates were characterised by their susceptibility to Proteinase K digestion and their relative mobility in SDS–PAGE gels (see Fig. 3). The TRA-Ribozyme conjugate migrated with an apparent molecular weight of about 160 kDa (Lane A) which was similar to that reported by us for TRA-oligonucleotide conjugates (Walker et al., 1995). To confirm the presence of the protein component (i.e. the non-radiolabelled antibody molecule), partial digestion with Proteinase K was performed. An intense band exhibiting a mobility similar to that of the unconjugated 37-mer ribozyme was observed (Lane B). This indicated the partial release of the radiolabelled ribozyme from the antibody molecule following Proteinase K digestion. In addition, a slower migrating weak band was also observed which represented a higher molecular weight product composed of ribozyme-conjugates with digested antibody product (Lane B). To rule out that the synthesised conjugate merely represented ribozyme adsorbed onto the antibody protein, the non-derivatized transferrin antibody was incubated with the radiolabelled-ribozyme as per the synthetic reaction and also analysed by SDS–PAGE (Lane C). The absence of a high molecular weight band corresponding to the conjugate excluded complexation by adsorption implying that the TRA-ribozyme conjugate was covalently coupled. Lane D shows the relative mobility of ‘free’ [^{32}P]-γ-dATP radiolabel used as a control marker for the appearance of nucleotide monomers. Overall the SDS–PAGE analysis was consistent with the proposed antibody-ribozyme synthetic product depicted in Fig. 2B.

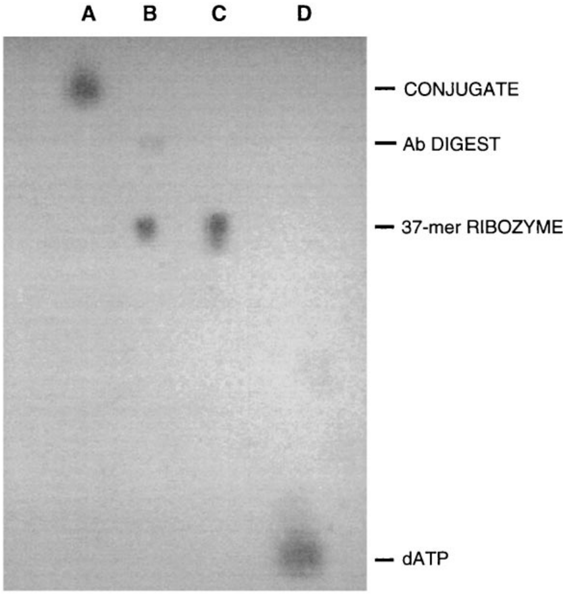

Fig. 3. Characterisation of the anti-transferrin receptor antibody-ribozyme conjugate. Lane A is the ribozyme-antibody conjugate. Lane B is the ribozyme-antibody conjugate incubated with Proteinase K. Lane C is a physical mix of the 37-mer internally radiolabelled ribozyme and the non-derivatised antibody. Lane D is the ‘free’ radiolabel ([^{32}P]-γ-dATP) used to internally label the ribozyme molecule.

The number of ribozyme molecules conjugated to the transferrin receptor antibody was quantified spectrophotometrically by analysis of three separately prepared batches of the TRA-ribozyme conjugate. On average, five or six ribozyme molecules were bound to each antibody molecule in the synthesis reaction. Previous studies have reported that approximately 10 molecules of a 20-mer antisense oligonucleotide became conjugated to an anti-transferrin receptor antibody when a similar conjugation reaction was performed (Walker et al., 1995). The lower conjugation ratio observed here is probably related to the larger size and defined ‘wishbone-like’ conformation of the 37-mer ribozyme.

3.2. Cell association studies

The cellular association of the TRA-ribozyme conjugate was examined in A431 tumour cells at fixed time intervals (30, 60 and 120 min). The cellular association of the TRA-ribozyme conjugate was significantly higher ($P < 0.001$) than that of the unconjugated ribozyme at all time points tested (see Fig. 4). Levels of cell associated TRA-ribozyme were approximately 3-fold higher than that of the unconjugated ribozyme after incubation periods up to 60 min. Furthermore, the level of association remained more than 2-fold higher than that of the unconjugated ribozyme after 2 h, despite the fact that levels of cell associated TRA-conjugate achieved a plateau level and did not significantly increase ($P = 0.069$) beyond 60 min.

We next attempted to investigate whether the improved cellular association of TRA-ribozyme conjugates was a direct result of transferrin receptor-mediated endocytosis (RME). RME is an energy dependant and saturable process which can be inhibited at reduced temperature. Therefore, cellular association of the TRA-ribozyme conjugate was compared at both 37 and 4°C. Results indicated that the mechanism of cellular uptake of the TRA-ribozyme conjugate was highly temperature dependant (see Fig. 5A). The percentage of TRA-conjugated ribozyme which became cell associated was reduced more than 5-fold ($P = 0.003$) at 4°C, when compared with results observed at 37°C. This indicated that the TRA-ribozyme conjugates were internalised via an energy-dependant process and is similar to that found for ribozyme alone (Fell et al., 1997). In addition, competition studies were performed in which the non-derivatised antibody was added to the culture media (at 5-molar excess). The cellular association of the TRA-ribozyme conjugate was inhibited by almost 60% ($P =$

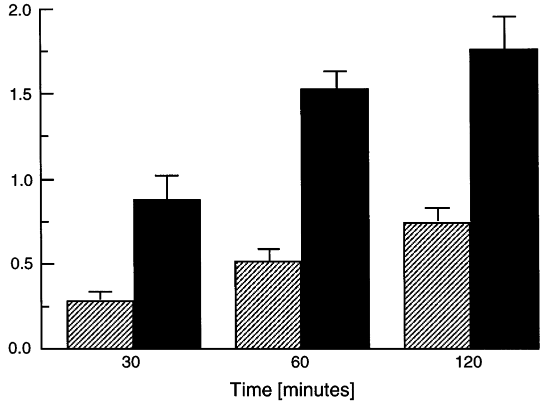

Fig. 4. Cell association (%) of TRA-Ribozyme conjugate (filled bars) in A431 cells as a function of time compared with an unconjugated ribozyme control (shaded bars). Experiments were performed at 37°C, $n = 4$, values represent mean ± S.D.

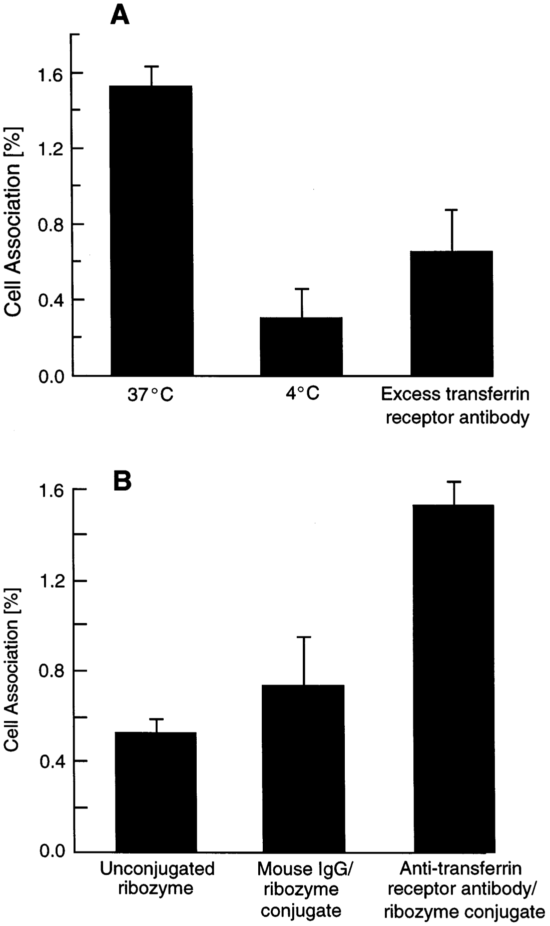

Fig. 5. A. Percentage cell association of TRA-conjugated ribozymes at 37 and 4°C after 1 h incubation and in the presence of a 5-fold excess of unconjugated TRA ($n = 4$, mean values ± S.D). B. Percentage cellular association of chimaeric ribozymes when conjugated to specific and non-specific antibody molecules compared with the unconjugated ribozyme control. ($n = 4$, mean values ± S.D).

0.004) in the presence of excess (unlabelled) non-derivatised antibody molecule. This indicated that the TRA-ribozyme conjugate and the 'free' antibody were in competition for binding sites on the transferrin receptor. Therefore, the specific involvement of the transferrin receptor in the cellular uptake of the TRA-ribozyme conjugate was implied. The partial inhibition observed could be due to a lack of binding site saturation by the competing antibody or more likely indicates that cellular uptake of TRA-ribozyme conjugates did not occur exclusively via transferrin receptor-mediated endocytosis. The latter explanation was further supported by the fact that ribozymes conjugated to a non-specific IgG were able to enter cells to some extent (see Fig. 5B), despite the fact that they would not be predicted to bind to any specific receptor on the surface of the A431 cells. The cellular association of the TRA-ribozyme conjugate was compared with that of the mouse anti-human IgG-ribozyme conjugate at equimolar antibody concentrations in A431 cells along with unconjugated ribozyme as a control (see Fig. 5B). Cells were incubated with each agent for 1 h in serum-free media at 37°C. Cellular association of the TRA-ribozyme conjugate was almost 2-fold higher ($P < 0.001$) than that of the mouse anti-human IgG-ribozyme conjugate. The cellular association of the anti-human IgG-ribozyme conjugate was slightly higher than that observed for the free ribozyme control after 1 h, however, the difference between mean levels of cell association was not statistically significant ($P = 0.095$).

These data indicated that conjugation of the ribozyme to the anti-transferrin receptor antibody could increase cellular uptake via a mechanism which was specifically related to the presence of the transferrin receptor antibody. By comparison, conjugation to mouse anti-human IgG did not significantly increase ribozyme uptake by A431 cells. This suggests that non-specific association of conjugated antibody molecules to cellular components was unlikely to account for the increase in cellular association observed with the TRA-ribozyme conjugate.

3.3. The effect of monensin on the cellular association of TRA-ribozyme conjugates

To further investigate the specific involvement of the transferrin receptor and to examine if interrupting vesicular trafficking could further improve cellular uptake of the TRA-ribozyme conjugate, the effects of the carboxylic acid ionophore monensin were examined. Monensin is known to raise the pH of endosomes (Mollenhauer et al., 1990) and also has been shown to specifically inhibit the recycling of transferrin (the natural ligand) and the transferrin receptor (Stein et al., 1984). The inhibition of transferrin receptor recycling is thought to occur via disruption of normal vesicular transport via the trans-Golgi network (Tartakoff, 1983). Pre-treatment and co-incubation with monensin increased the percentage of cell associated TRA-ribozyme conjugate by almost 40% ($P = 0.0049$) after 30 min incubation (see Fig. 6). In comparison, monensin did not significantly ($P = 0.0681$) affect cellular association of unconjugated ribozyme during the same time period. These findings suggest that ribozyme delivery via transferrin receptor-mediated pathway is further enhanced upon monensin treatment. It also suggests that naked ribozymes are not trafficked via the monensin-sensitive vesicular transport pathways, although this requires further study.

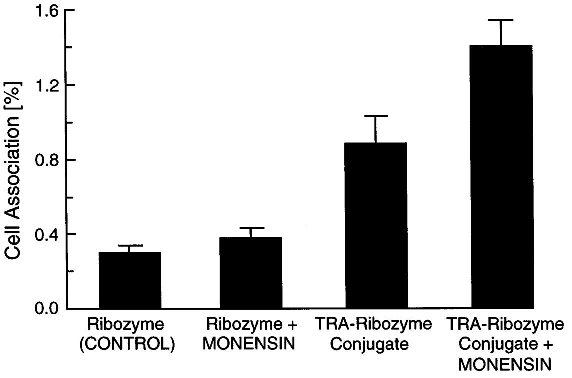

Fig. 6. The effect of monensin on the cellular association of TRA-conjugated ribozyme and unconjugated ribozyme after 30 min incubation at 37°C. (For monensin treated samples $n = 3$, for other samples $n = 4$, values are mean ± S.D).

Our data suggest that it may be possible to use TRA-conjugates to improve delivery of ribozymes to cells expressing high levels of transferrin receptors such as many types of tumour cells (Trowbridge and Omary, 1981; Citro et al., 1992; Shah and Shen, 1995) and the capillary microvessel endothelial cells which comprise the blood brain barrier (BBB) (Friden, 1993). Recent pharmacokinetic studies indicate that unconjugated (chemically stabilised) ribozymes are effectively excluded from the brain following systemic delivery (Desjardins et al., 1996). Hence, transferrin receptor-mediated delivery of ribozymes may offer an effective strategy for CNS delivery of these pharmaceutically challenging molecules. It must be noted, however, that the release of the ribozyme from the antibody conjugate, following internalisation by cells, could potentially limit the efficacy of ribozymes delivered to cells via this strategy. In this report, a stable thioether linkage was used for the conjugation of the ribozyme to the antibody. However, to facilitate cellular delivery a cell-labile linkage such as a disulphide bridge could be used to attach the ribozyme molecule to the antibody, and thus, facilitate intracellular release of ribozymes. Another factor to consider is the fate of TRA-ribozyme conjugates following cellular internalisation by RME. Even the enhanced uptake of conjugates in the presence of monensin, may still result in ribozyme accumulation within vesicular compartments whereas ribozymes would almost certainly have to penetrate sites within the nucleus or cytoplasm in order to hydrolyse target mRNAs. The intracellular trafficking of the TRA-ribozyme conjugates in the presence and absence of monensin requires further study to identify the ultimate fate of the ribozyme and/or the antibody conjugate. However, if escape from intracellular vesicles became a limitation to the use of TRA-ribozyme conjugates, the inclusion of vesicle disrupting agents such as fusogenic peptides (see Bongartz et al., 1994) or association with helper lipids within the conjugate could represent potential means of improving cytoplasmic penetration.

There are recent suggestions, however, that uptake of nucleic acids in vivo may be more efficient than appears in cell culture (e.g. see Akhtar and Agrawal, 1997; Akhtar, 1998). This is further supported by in vivo studies with ribozymes where biological activity has been achieved with naked ribozymes (Lyngstadaas et al., 1995; Sioud, 1996; Flory et al., 1996; Jarvis et al., 1996). In this scenario, the improved delivery of ribozymes using TRA-conjugates may offer a method of reducing dose requirements of these relatively expensive biologicals. Furthermore, localised administration to tumours may also facilitate drug targeting to transferrin receptor-expressing tumour cells. However, a more detailed understanding of the mechanisms of ribozyme uptake and delivery in vivo in specific disease conditions and in the presence of circulating proteins is required before the full potential of transferrin mediated delivery can be realised.

Acknowledgements

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