Amino Acids (1993) 4: 237–248

Amino
Acids

Antifibrotic effect of a proline analogue delivered in liposomes to cells in culture

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Accepted September 2, 1992

Summary. Proline analogues inhibit procollagen triple helix formation and are antifibrotic in vivo. Efficacy of the proline analogue *cis*-4-hydroxy-L-proline (cHyp) on vascular collagen accumulation is improved by in vivo delivery in liposomes. This effect may be due to local release of drug from liposomes taken up by vascular endothelium. To test this postulate, we used a co-culture system to assess the antifibrotic effect of cHyp in liposomes taken up by endothelium (upper well) by measuring inhibition of growth of smooth muscle cells and fibroblasts (lower well). We also studied whether release of cHyp was prolonged in poly(ethyleneglycol) (PEG)-conjugated liposomes compared to liposomes not conjugated with PEG (control liposomes). In fibroblasts, free (unencapsulated) cHyp (1 mg/ml) added to the upper well inhibited growth for 3 days; an equivalent dose of cHyp in control liposomes inhibited growth for 4 days. cHyp in PEG-liposomes produced greater growth inhibition than cHyp in control liposomes. cHyp in liposomes did not inhibit growth of smooth muscle cells more than free cHyp. Washing free cHyp from endothelium after 1 day incubation restored growth of smooth muscle cells whereas washing liposomes containing cHyp failed to restore cell growth. These results suggest that liposomes enhance drug efficacy of cHyp by prolonging the release of drug from endothelium.

Keywords: Amino acids – Poly(ethyleneglycol) – Fibrosis – Collagen – *cis*-4-hydroxy-L-proline – Drug delivery – Endothelium – Vascular biology

Introduction

Proline analogues have been used in studies of the synthesis and secretion of procollagen and to study cell-matrix interactions (Wilson and Hatfield, 1984).

Their mechanism of action following incorporation into peptide linkages of procollagen is to reduce thermal stability of the protein by interfering with formation of triple-helical conformation (Uitto and Prockop, 1975) leading to intracellular accumulation (Uitto et al., 1975) and accelerated intracellular degradation (Berg et al., 1980) of procollagen. The net result is decreased secretion of procollagen and reduced accumulation of collagen in the extracellular space. Based on these biochemical properties, proline analogues have been used in models of fibrosis where reduction in synthesis of collagen is desirable (Fuller, 1981a, 1981b; Uitto et al., 1982).

Chronic administration of proline analogues is toxic to animals (Geismar et al., 1988), and one method of reducing drug toxicity is to deliver the drug in a carrier vehicle to the tissue undergoing fibrosis. We have shown that the proline analogue *cis*-4-hydroxy-L-proline (cHyp) prevents collagen accumulation in hypertensive blood vessels and that toxicity is reduced by intravenous delivery of cHyp in liposomes or lipid bilayers that enclose an aqueous compartment (Poiani et al., 1992). Blood vessels contain a single layer of endothelial cells which are capable of taking up particles from blood (Ryan, 1988). Following intravenous delivery, it is probable that liposomes containing cHyp are taken up by endothelium and cHyp is locally released to nearby smooth muscle cells and fibroblasts, thereby inhibiting collagen accumulation in the blood vessel wall (Poiani et al., 1992).

We hypothesize that delivery of cHyp in liposomes to endothelium results in more sustained inhibition of collagen production by vascular cells than the free (unencapsulated) agent. To test this postulate, we used an in vitro system in which liposomes containing cHyp were added to the upper well of a co-culture system, and inhibition of growth of smooth muscle cells or fibroblasts in the lower well was measured. Inhibition of cell growth by cHyp was used as an index of antifibrotic activity based on the principle that cells grown on plastic dishes must secrete a collagenous protein to attach and grow (Kao and Prockop, 1977). We assessed growth inhibition by cHyp released from two types of cationic liposomes, control liposomes and liposomes conjugated with poly(ethyleneglycol) (PEG). PEG is a dehydrating polymer that withdraws water molecules from cell membranes, forcing close contact of lipid membranes. Following intravenous injection, PEG-conjugated liposomes have been shown to persist in the blood for longer periods of time than standard liposomes (Allen and Chonn, 1987; Allen et al., 1991; Blume and Cevc, 1990; Gabizon and Papahadjopoulos, 1988; Klivanov et al., 1990; Woodle et al., 1990). Conventional liposomes fail to stay in the circulation for more than a few hours because of binding to plasma protein components which enhance removal by the mononuclear-phagocytic system (Poste et al., 1982). The longer circulating time of PEG-conjugated liposomes is presumed to be due to decreased accessibility of plasma proteins to the liposome surface, thus delaying phagocytosis (Blume and Cevc, 1990). Thus, PEG-conjugated liposomes could enhance electrostatic interaction with the negatively charged endothelial surface by preventing quenching of cationic liposomes that would result from coating with anionic serum proteins.

Methods

Materials

Materials were L-α-dipalmitoylphosphatidylcholine (780 g/mol) (Avanti Polar Lipids, Birmingham, AL); penicillin, streptomycin, gentamicin, *trans*-4-hydroxy-L-proline (tHyp), ascorbate, cholesterol (386.6 g/mol), stearylamine (269.5 g/mol) and trypan blue (Sigma Chemical Co., St. Louis, MO); *cis*-4-hydroxy-L-proline (cHyp) (Peptide International, Louisville, KY); rabbit anti-factor VIII antibody and FITC-goat-anti-rabbit antibody (Calbiochem Corp., LaJolla, CA); collagen (type I from rat tail) (Collaborative Biomedical Products, Bedford, MA); phosgene solution in toluene (20% v/v) (Fluka Chemical Corp., Ronkonkoma, NY); triethylamine (Aldrich Chemical Co., Inc., Milwaukee, WI); N-hydrosuccinimide (Fisher Scientific, Pittsburgh, PA); [^{14}C]-L-proline (260 mCi/mM) (New England Nuclear Co., Boston, MA); medium 199 with Earle's salts and fetal bovine serum (FBS) (ICN Biomedicals, Inc., Costa Mesa, CA); trypsin-EDTA (Gibco-BRL, Grand Island, NY); liquiscent (National Diagnostics, Inc., Somerville, NJ); methoxy polyethylene glycol (Mw 5,000) (Union Carbide Co., Danbury, CT). All chemicals were analytical grade; solvents used in the synthesis were HPLC grade.

Chemical analysis

FT-IR spectra were obtained on a spectrometer (model 100, Mattson Cygnus, Mattson Instrument Co., Madison, WI). ^{1}H and ^{13}C NMR spectra were recorded on a spectrometer (model XL-200, Varian Analytical Instruments, Sunnyvale, CA) using $CDCl_3$ as solvent and tetramethylsilane as internal reference. The amount of active carbonate groups present after N-hydroxysuccinimide activation of PEG was determined by non-aqueous titration established by Kalir and associates (1974).

Activation of methoxy poly(ethyleneglycol) and preparation of PEG-modified stearylamine

Methoxy poly(ethyleneglycol)-N-succinimidyl carbonate (SC-PEG) (Fig. 1) was prepared as described by Zalipsky and associates (1991). SC-PEG (16 g, 3.1 mmol) was dissolved in

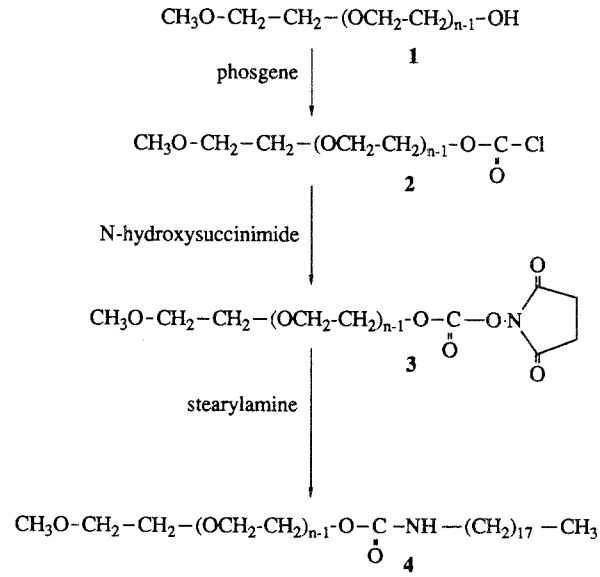

Fig. 1. Scheme showing the synthetic steps for the preparation of the PEG derived stearylamine. **1** Methoxy PEG (Mw = 5000). **2** Methoxy PEG chloroformate. **3** Methoxy PEG succinimidyl carbonate. **4** Methoxy PEG modified stearylamine

methylene chloride (200 ml) (Fig. 1). Stearylamine (1.25 g, 4.65 mmol) was added to this solution, the reaction mixture was stirred at room temperature for 24 h, filtered, concentrated to a 10 ml volume and poured into cold ether. After cooling at 4°C, the crude material was collected by filtration and purified by precipitation with isopropanol twice. Recovery: 11.5 g (70%). FT-IR (film on NaCl, cm^{-1}): characteristic bands at 2883 (CH_2) and 1715 (C = O, urethane). ^{13}C NMR ($CDCl_3$): δ 156 (C = O of urethane); 68.3–70.2 (several peaks due to PEG-OCH_2); 58.5 (CH_3O); 40.6 (-N-CH_2); 13.9–22.3 (several peaks due to stearyl CH_2); and 13.91 (stearyl CH_3).

Preparation and characterization of liposomes

Unilamellar, positively charged, small phospholipid vesicles (control liposomes) were prepared by reverse phase evaporation by the method of Szoka and Papahadjopoulos (1978) as modified by Turrens and associates (1984). Liposomes were synthesized from L-α-dipalmitoylphosphatidylcholine, cholesterol and stearylamine in a molar ratio of 14:7:4 as previously described (Poiani et al., 1992). The PEG-conjugated liposomes were prepared with a 5 molar percent substitution of PEG-modified stearylamine for unconjugated stearylamine. All batches were used within 4 h of preparation. Lipid phosphorus was quantitated using the ammonium molybdate method of Chen et al. (1956) as modified by Ames and Dubin (1960).

Liposome diameter was estimated by a single beam fluorescent activated cell sorter (Epic 752 Dye Laser System, Coulter Electronics, Hialeah, FL) as previously described (Poiani, et al., 1992) and ranged between 100 and 200 nm. Entrapment was estimated as the percentage of [^{14}C]-L-proline sequestered in liposomes and was 51 ± 6% (n = 11) for control liposomes and 55 ± 5% (n = 8) for PEG-conjugated liposomes. Structural integrity of liposomes, assessed by retention of [^{14}C]-L-proline, remained constant during storage at 4°C for 21 days for both liposome preparations.

Cell cultures

Pulmonary artery endothelial cells were obtained from calf pulmonary arteries as previously described (Poiani et al., 1992). Cells were placed in medium 199 containing 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, pH 7.4, and incubated at 37°C under 95% O_2, 5% CO_2. Medium was not changed during the first week and thereafter medium was changed twice weekly. Endothelial cells were identified by their characteristic cobblestone appearance in culture and the presence of factor VIII-related antigen by immunofluorescence (Jaffe et al., 1973). Fetal rat aortic smooth muscle cells and fetal rat lung fibroblasts (American Type Tissue Culture, Rockville, MD) were plated separately (1×10^6 cells) in the same medium containing 50 μg/ml sodium ascorbate on 25 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) and grown under 95% O_2, 5% CO_2. Cells were removed by trypsin-EDTA, split (2:1), and used after the fourth passage.

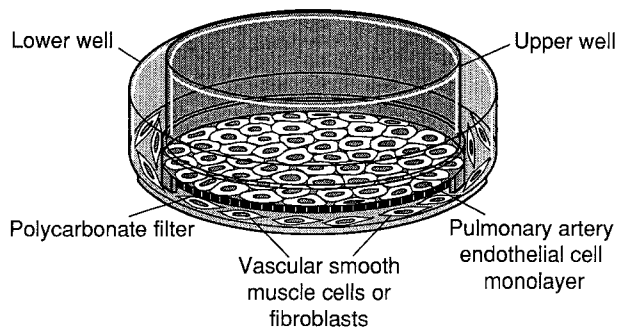

Fig. 2. Scheme of endothelial cell-smooth muscle cell/fibroblast co-culture system

For the co-culture system, an upper well consisting of a 30 mm^2 polycarbonate dish containing a porous filter (0.4 μm pore size, Millicell-CM, Millipore, Bedford, MA) was placed above a 35 mm^2 polystyrene dish (Corning) (Fig. 2). Endothelial cells (1×10^5 cells/dish) were placed in the upper well and grown in medium 199 containing the constituents mentioned above. After one day, fresh medium was added, and the cells were grown to confluence in 3 days. Confluence was assessed by the electrical resistance across the monolayer measured by a current voltage clamp generator (Millicell-ERS, Millipore). Smooth muscle cells or fibroblasts were seeded (1×10^5 cells/dish) in the lower wells and grown in medium 199 containing the above constituents except the concentration of FBS was 0.5%. After one day, medium was changed to fresh medium containing 10% FBS.

Assay of antifibrotic effect of cHyp

Inhibition of cell growth in culture was used as an index of antifibrotic effect of cHyp based on the principle that cells in culture require the secretion of collagenous proteins to attach and grow on plastic (Kao and Prockop, 1977). Specificity for collagen deposition can be shown by lack of growth inhibition by cHyp when cells are grown on collagen-coated plates (Kao and Prockop, 1977). The antifibrotic assay was performed by measuring growth inhibition by cHyp of smooth muscle cells or fibroblasts grown alone or in the co-culture system. Cells grown on collagen-coated plates in the presence of cHyp were used to check for specificity of inhibition of collagen secretion.

To assay for an inhibitory effect of free (unencapsulated) cHyp, cHyp (0.25, 0.50, and 1.0 mg/ml) was added to cultures of smooth muscle cells and fibroblasts grown on plastic dishes in medium containing 10% FBS, and cells were counted manually at 1, 2, 3 and 4 days. Results were compared to cells grown in the presence of the same concentrations of the amino acid tHyp and cells grown in the presence of cHyp on plates precoated with type I collagen extracted from rat tail. Cell viability was checked by exclusion of trypan blue dye (0.4% w/v). Results of cell counts for each cell type on each day were compared among cells grown on plastic in the presence of cHyp and tHyp and cells grown on collagen-coated plates in the presence of cHyp.

The co-culture system was used to compare growth inhibition of smooth muscle cells or fibroblasts in the lower well by free cHyp or cHyp in liposomes added to the endothelial cell monolayer in the upper well. The upper well containing a confluent monolayer of endothelial cells was placed on top of the lower well at the time when growth of the smooth muscle cells or fibroblasts was stimulated by changing the medium to a high (10%) concentration of FBS (referred to as day zero). The test substance was added to the upper well at this time, and growth of the cells in the lower plate was measured at 1, 2, 3 and 4 days. The following test substances were added to the upper well: free cHyp (1 mg/ml); control liposomes (~ 1.4 μmol phospholipid in ~ 40 μl) containing 1 mg/ml cHyp; and PEG-conjugated liposomes containing 1 mg/ml cHyp.

To determine whether growth inhibition was sustained after removal of the test substance, other experiments were done in which free cHyp, control liposomes containing cHyp and PEG-conjugated liposomes containing cHyp were added to the upper well at day zero, and the monolayer washed $\times 3$ with fresh medium at day 1 to remove the test substance. Cell counts were made at days 1, 2, 3 and 4, and results were compared to experiments in which the test material was not removed.

For all experiments, four cultures were performed for each test substance, and the average results of the experiments were compared for tHyp, free cHyp, control liposomes containing cHyp, and PEG-conjugated liposomes containing cHyp.

Statistical analysis

Mean $\pm$ SEM for each group were obtained. Data were analyzed by using an ANOVA procedure with SAS® software (SAS®) followed by Duncan's post-hoc test (Duncan, 1975). A P value of 0.05 was considered significant.

Results

Effects of free cHyp on single cell cultures

Free cHyp (1 mg/ml) inhibited growth of fibroblasts for 3 days (Fig. 3A) and smooth muscle cells for 4 days (Fig. 3B) compared to cells grown in the presence of tHyp (1 mg/ml). Doses of 0.25 and 0.5 mg/ml had no inhibitory effect (data not shown). *Cis*-4-hydroxy-L-proline (1 mg/ml) had no inhibitory effect on either cell type grown on collagen-coated plates (Fig. 3). Cell viability was >98% in all experiments.

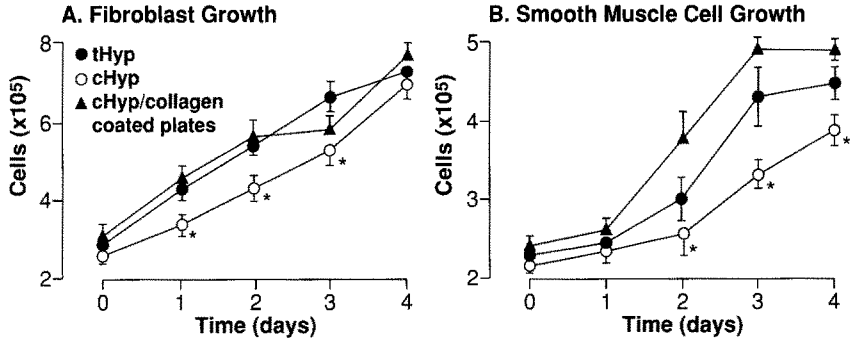

Fig. 3. Graphs showing growth of single cells in culture. **A** Fibroblast growth. **B** Smooth muscle cell growth. Growth of quiescent cells was stimulated by addition of 10% fetal calf serum (day zero), and cell counts measured. *tHyp* cells grown on plastic dishes in presence of 1 mg/ml *trans*-4-hydroxy-L-proline (tHyp); *cHyp* cells grown on plastic dishes in presence of 1 mg/ml *cis*-4-hydroxy-L-proline (cHyp); *cHyp/collagen coated plates* cells grown on collagen coated plates in the presence of 1 mg/ml cHyp. Data points, mean; brackets, ±SEM; n = 4. *$p < 0.05$ compared with control

Effects of free cHyp and cHyp in control liposomes on co-cultures

Free cHyp (1 mg/ml) inhibited growth of fibroblasts in co-culture for 3 days; there was no growth inhibition on day 4 (Fig. 4A). Smooth muscle cell growth was inhibited for 4 days (Fig. 4B). Control liposomes containing cHyp inhibited growth of both cell types for 4 days (Fig. 4). There was no difference in growth inhibition with cHyp in control liposomes compared to free cHyp for either cell type (Fig. 4). There was no inhibition by cHyp or cHyp in control liposomes for growth by either cell type grown on collagen-coated plates (data not shown).

Effects of cHyp in control liposomes and PEG-liposomes on co-cultures

PEG-conjugated liposomes containing cHyp (1 mg/ml) caused inhibition of growth of fibroblasts on days 2–4 and caused a greater inhibition of growth than control liposomes on day 4 (Fig. 5A). There was no apparent difference in inhibition of growth of smooth muscle cells by PEG-conjugated liposomes compared to control liposomes on any day (Fig. 5B). There was no inhibition by cHyp in control or PEG-conjugated liposomes for either cell type grown

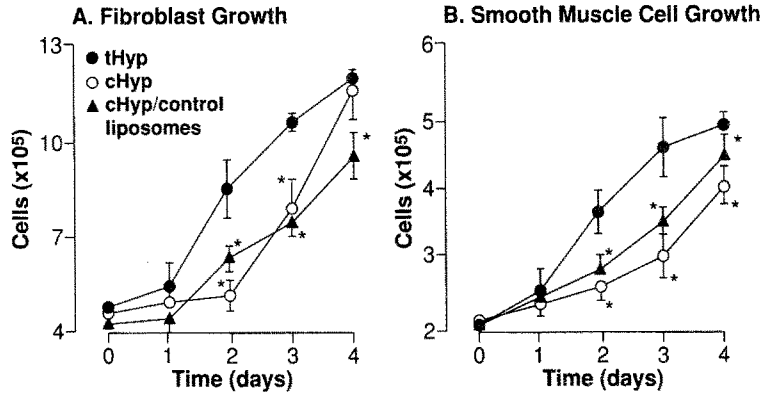

Fig. 4. Graphs showing growth of cells in co-culture system. **A** Fibroblast growth. **B** Smooth muscle cell growth. *cHyp/control liposomes* cHyp encapsulated in control liposomes. Cells were grown on plastic dishes in presence of 1 mg/ml free tHyp, 1 mg/ml cHyp, or 1 mg/ml cHyp in control liposomes. Format same as Fig. 3

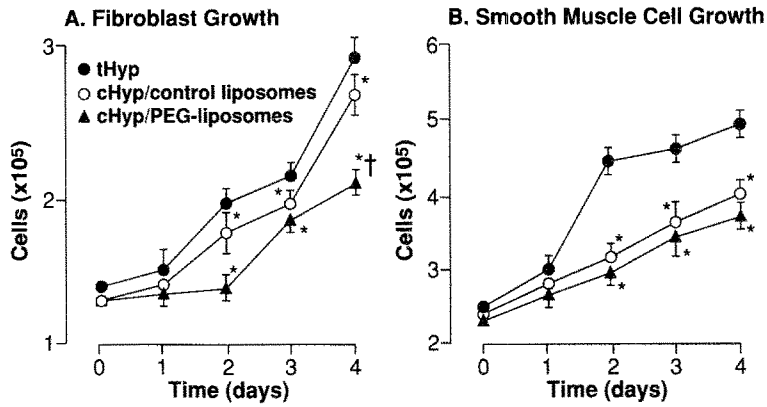

Fig. 5. Graphs showing growth of cells in co-culture system. **A** Fibroblast growth. **B** Smooth muscle cell growth. *cHyp/PEG-liposomes* cHyp encapsulated in poly(ethyleneglycol) conjugated liposomes. Cells were grown on plastic dishes in presence of 1 mg/ml tHyp, 1 mg/ml cHyp/control liposomes, or 1 mg/ml cHyp/PEG-liposomes. † $p < 0.05$ compared to cHyp/control liposomes. Format otherwise same as Fig. 3

on collagen-coated plates (data not shown). Cell viability was >98% in all experiments.

Effects of cHyp incubated for 24 hours on subsequent cell growth

Addition of free cHyp, cHyp in control liposomes and PEG-conjugated liposomes (each 1 mg/ml) produced inhibition of fibroblast growth on day 2 compared to growth of cells incubated with free tHyp (Fig. 6A). Washing the cells after 1 day produced stimulation of growth of the free cHyp treated fibroblasts (Fig. 6A). Inhibition of growth persisted from days 2–4 in cells treated with control liposomes and PEG-conjugated liposomes containing cHyp (Fig. 6A). Addition of free cHyp, cHyp in control liposomes and PEG-conjugated lipo-

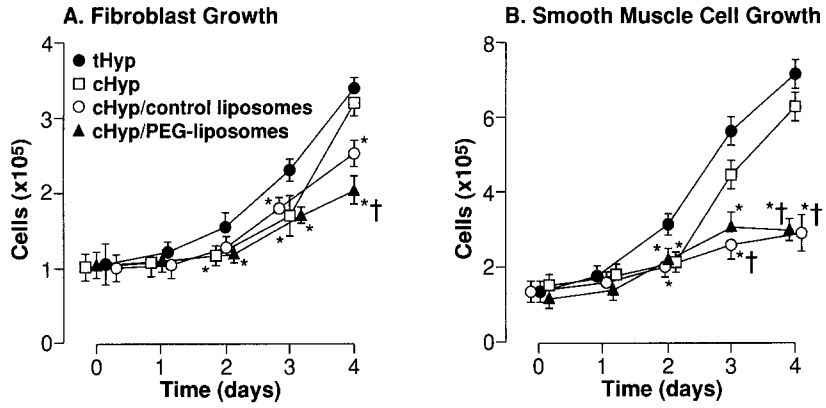

Fig. 6. Graphs showing growth of cells in co-culture system before and after washing of lower well to remove cHyp. **A** Fibroblast growth. **B** Smooth muscle cell growth. Cells were grown on plastic coated dishes in the presence of 1 mg/ml tHyp, 1 mg/ml cHyp, 1 mg/ml cHyp in control liposomes or 1 mg/ml cHyp in PEG-liposomes. Cells were washed at day 1, and cell number measured on following days. † $p < 0.05$ compared to cHyp. Format otherwise same as Fig. 3

somes (each 1 mg/ml) inhibited smooth muscle cell growth at 2 days (Fig. 6B). Washing the cells after 1 day produced growth of cells treated with free cHyp after 2 days, but growth inhibition persisted in cells treated with cHyp in control liposomes and PEG-conjugated liposomes through day 4 (Fig. 6B). There was no inhibition by cHyp in any form for either cell type grown on collagen-coated plates (data not shown). Cell viability was >98% for all experiments.

Discussion

The relative specificity of proline analogues for inhibiting collagen synthesis has been used to reduce synthesis of collagen in models including tendon repair (Bora et al., 1972), liver cirrhosis (Rojkind, 1973), pulmonary fibrosis (Riley et al., 1980, 1981, 1984) and pulmonary hypertension (Kerr et al., 1987; Poiani et al., 1990, 1992). In the present experiment, we studied the effects of free and encapsulated cHyp in a co-culture system which was designed to simulate the anatomic relationship of endothelial cells to collagen producing smooth muscle cells and fibroblasts in the walls of lung vessels. Our results show that administration of cHyp to fibroblasts and smooth cells in culture inhibit cell growth. The inhibitory effect was dependent on secretion of collagenous proteins since cHyp had no effect on cells grown on dishes precoated with collagen. These results suggest that cHyp interfered with secretion of collagenous proteins which is essential for cells to attach and proliferate in culture (Kao and Prockop, 1977).

In the blood vessel wall, drugs delivered intravenously must pass through the endothelium to reach smooth muscle cells and fibroblasts, the major collagen-producing vascular cells (Mayne, 1987). Free cHyp added to the upper well of the co-culture system inhibited growth of both cell types in a manner similar to the single cell culture system. This is probably due to free diffusion of the imino acid analogue (Mw 131) through cells.

We observed that growth inhibition of fibroblasts by cHyp in both single and co-culture was limited to three days. The restricted period of growth inhibition was likely due to a reduced concentration of cHyp to a level too low to inhibit procollagen processing, possibly due to increased number of collagen producing cells relative to the concentration of cHyp. Smooth muscle cell growth was inhibited for at least 4 days, a longer period than fibroblast growth inhibition. The difference in growth inhibition between the two cell types may possibly be because smooth muscle cells grew more slowly than fibroblasts or that smooth muscle cells secrete less collagen per cell than fibroblasts.

The important observation in this study is that delivery of an equivalent dose of cHyp in liposomes produced inhibition of fibroblast growth for four days compared to three days for free cHyp. Lipsomes are internalized by endothelial cells in culture by a process which involves endocytosis (Pagano, 1978). We have previously shown that control liposomes are taken up by pulmonary artery endothelial cells with a half-life of ~ 25 minutes and that a maximal uptake of $\sim 5\%$ of total dose occurs within 2 hours (Poiani et al., 1992). The more prolonged effect of cHyp in liposomes compared to free cHyp is probably due to the greater concentration of cHyp retained in liposomes in endothelial cells in close proximity to the collagen-producing fibroblasts. Retention of liposomes and release of cHyp by endothelial cells is further suggested by the continued inhibitory effect on cell growth by encapsulated cHyp after washing liposomes not taken up by endothelium.

We observed that PEG-conjugated liposomes produced more prolonged growth inhibition of fibroblasts in co-culture than control liposomes. In these in vitro experiments, serum was present in the medium, it is possible that serum proteins bind to the surface of control liposomes and decrease their uptake by endothelium. The presence of PEG on the liposome surface may have improved uptake in endothelium, possibly by membrane-disrupting activity in a way analogous to PEG-conjugated bolaphiles (single-chain surfactants bearing a polar head group at each end of a hydrophobic segment) (Jayasuriya et al., 1990). The enhanced uptake may have caused a greater concentration of cHyp near the target cells, thus causing more prolonged antifibrotic effects. Another explanation is that incorporation of PEG into the liposome bilayer increased the rigidity of the carrier membrane, thereby decreasing permeability of the bilayer and slowing drug elimination from the liposome. Whatever the mechanism, our results suggest that PEG-conjugated liposomes offer potential advantages in drug delivery to endothelial cells compared to control liposomes. Therefore our future work will focus on the use of PEG-conjugated liposomes to deliver polymers of cHyp (Gean et al., 1992) to pulmonary endothelial cells to prolong the antifibrotic activity of cHyp in lung vessels.

In conclusion, our results show that cHyp in liposomes is taken up by endothelial cells and can act as a depot for prolonged delivery of antifibrotic agents to adjacent smooth muscle cells and fibroblasts. In addition, PEG-conjugated liposomes produce a more prolonged delay in release of cHyp than control liposomes. These observations may be important in designing strategies to treat sclerosing vascular disorders by targeting antifibrotic agents to blood vessel walls in drug carrier systems. Our results suggest that studies are needed

to define the mechanisms of uptake of PEG-conjugated liposomes into endothelial cells and the effects on binding with serum proteins. Additional studies into the molecular mode of action of PEG containing drug carriers also need to be conducted.

Acknowledgements

This work was supported by a Glaxo Cardiovascular Discovery Grant, U.S. Public Health Service Grants HL24264, HL44081, and HL07467, the Barbara Wallace Cornwall Respiratory Research Laboratory, the John M. Kent Pulmonary Fibrosis Fund, the American Heart Association/New Jersey Affiliate and the Medical Research Service of the Department of Veterans Affairs. One of the authors (JK) acknowledges the support of an NIH Research Career Development Award GM00550.

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Received June 22, 1992