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A Synthetic Heparin-Mimicking Polyanionic Compound Binds to The LDL Receptor-Related Protein and Inhibits Vascular Smooth Muscle Cell Proliferation

Miriam Benezra,[1] Tikva Vogel,[2] Shmuel A. Ben-Sasson,[3] Amos Panet,[4] Ephraim Sehayek,[5] Mayson Al-Haideiri,[6] Richard J. Decklbaum,[6] and Israel Vlodavsky[1]*

[1]Department of Oncology, Hadassah University Hospital, Jerusalem, Israel
[2]Biotechnology General Ltd, Rehovot, Israel
[3]Department of Experimental Medicine and Cancer Research, the Hebrew University-Hadassah Medical School, Jerusalem, Israel
[4]Department of Virology, the Hebrew University-Hadassah Medical School, Jerusalem, Israel
[5]Institute of Lipid and Atherosclerosis Research, The Haim Sheba Medical Center, Tel Hashomer, Israel
[6]Institute of Human Nutrition and Department of Pediatrics, Columbia University, Jerusalem, Israel

Abstract A synthetic heparin-mimicking polyaromatic anionic compound RG-13577 (polymer of 4-hydroxyphenoxy acetic acid and formaldehyde ammonium salt, Mr∼5800) exhibits specific binding to vascular smooth muscle cells (SMCs) and inhibits their proliferative response to growth promoting factors. Receptor binding of ^{14}C-RG-13577 was efficiently competed by apolipoprotein E3 (apoE), lactoferrin, and the LRP (LDL receptor-related protein) receptor associated 39 kDa protein (RAP). Unlike cell surface binding of apoE, binding of RG-13577 to SMCs was not affected by heparin, heparan sulfate degrading enzymes, or low density lipoprotein (LDL). Moreover, wild-type and heparan sulfate-deficient Chinese hamster ovary (CHO) cells, as well as normal- and LDL receptor negative- human skin fibroblasts bind RG-13577, but not apoE, to a similar extent. On the other hand, homozygous mouse embryonic fibroblasts deficient in the LDL receptor-related protein (LRP) expressed a markedly reduced binding of RG-13577 as compared to normal mouse embryonic fibroblasts. These results indicate that RG-13577 and related compounds bind to the LRP receptor on the surface of vascular SMCs. Addition of lactoferrin to cultured SMCs protected the cells against the antiproliferative effect of compound RG-13577, suggesting that this inhibition is mediated by RG-13577 binding to LRP receptors on the SMC surface. Altogether, we have identified a series of synthetic polyaromatic anionic molecules that exhibit specific binding to LRP and therby exert an antiproliferative effect on vascular SMCs. These compounds are applied to suppress SMC proliferation associated with restenosis and accelerated atherosclerosis. J. Cell. Biochem. 81:114–127, 2001.

Key words: heparin mimicking compounds; antiproliferative activity; cell surface receptors; heparan sulfate; apolipoprotein E; LDL receptor-related protein; lactoferrin

Abbreviations used: SMCs, smooth muscle cells; CHO, Chines hamster ovary; HS, heparan sulfate; HSPG, heparan sulfate proteoglycans; DMEM, Dulbecco's modified Eagle's medium; LRP, LDL receptor-related protein; LDL, low density lipoprotein; apoE, apolipoprotein E3; RG-13577, polymer of 4-hydroxyphenoxy acetic acid and formaldehyde ammonium salt.

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*Correspondence to: Israel Vlodavsky, Department of Oncology, Hadassah Hospital, P.O.B 12000, Jerusalem, 91120, Israel.

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Proliferation of arterial smooth muscle cells (SMCs) and accumulation of cholesterol-rich lipoproteins are basic events in the pathogenesis of arteriosclerosis [Fuster et al., 1992; Ross, 1993]. Under physiological conditions, the majority of arterial SMCs remain in the G_0 phase and cell growth is controlled by a balance between endogenous proliferation-stimulating and proliferation-inhibiting factors. Initiation and progression of atherosclerotic lesions are characterized by synchronized events that include adhesion, migration, and uncontrolled

proliferation of cells. These alterations are dependent on growth factors, lipoproteins, and components of the extracellular matrix (ECM) [Fuster et al., 1992; Ross, 1993]. Among the growth factors, are platelet-derived growth factor (PDGF) [Ferns et al., 1991], basic fibroblast growth factor (bFGF) [Linder et al., 1991], interleukin-1 (IL-1) [Loppnow and Libby, 1990], and thrombin [Bar-Shavit et al., 1993], produced by macrophages and platelets, as well as by the vascular endothelium and SMCs. These cells also produce enzymes (e.g. elastase, collagenase, heparanase) that digest various constituents of the ECM and thereby release bFGF and possibly other growth factors (i.e., TGF-β) that are stored in basement membranes and ECM [Vlodavsky et al., 1993].

Several classes of lipoproteins take place in the formation of arteriosclerotic plaques. An integral protein of some of these particles is apolipoprotein E (apoE) that also binds with high affinity to heparin and heparan sulfate proteoglycans (HSPG) [Zhong-Sheng et al., 1993; Al-Haideri et al., 1997; Mahley and Huang, 1999]. ApoE participates in cholesterol metabolism through its high affinity interaction with cell surface receptors including the LDL receptor and the LDL receptor-related protein (LRP) [Krieger and Herz, 1994; Willnow, 1999, Herz et al., 2000]. ApoE also exhibits other biological activities that are not obviously related to lipid transport. For example, synthetic apoE peptides and apoE-containing lipoproteins are potent suppressors of lymphocyte activation by mitogens and antigens [Cardin et al., 1988; Vogel et al., 1994]. ApoE is a potent inhibitor of vascular endothelial and kaposis sarcoma cell proliferation [Vogel et al., 1994]. It also competes with bFGF for binding to HSPG and thus functions as an antiangiogenic molecule [Vogel et al., 1994]. It is conceivable that apoE and other molecules that interfere with the growth-promoting activity of heparin-binding growth factors such as bFGF and heparin-binding EGF (HB-EGF) may attenuate the progression of the atherogenic process [Mahley and Huang, 1999; Ishigami et al., 2000]. Among these molecules are unique species of heparin and heparan sulfate that inhibit the proliferation of vascular SMCs both in vitro and in vivo [Castellot et al., 1986; Schmidt et al., 1992].

In a search for synthetic and better defined heparin-like molecules, we have recently identified a series of negatively charged aromatic compounds (e.g., polyhydroxyphenoxy acetic acid) that mimic many of the biological activities of heparin [Benezra et al., 1992, 1994]. These non-sulfated anionic compounds, which include synthetic dye polymers (e.g., aurintricarboxylic acid) [Cushman et al., 1991; Regan et al., 1993], compete with heparin and heparan sulfate on bFGF binding [Miao et al., 1997], prevent vessel wall deposition of circulating bFGF [Medalion et al., 1997] and revert the transformed phenotype and autocrine growth of bFGF-transfected cells [Benezra et al., 1992]. In a recent study, we have demonstrated that these synthetic polyanionic compounds efficiently inhibit proliferation of vascular SMCs [Benezra et al., 1994]. This antiproliferative activity was attributed to their saturable binding to specific cell surface receptors. Surprisingly, this binding was efficiently competed by apoE, but there was no effect of heparin, heparan sulfate and a variety of growth factors and ECM components [Benezra et al., 1994]. These results raised the possibility that apoE and the newly identified polyanionic molecules exert their antiproliferative effect through binding to a common cell surface receptor. At least three cell surface components (i.e., HSPG, LDL receptor, LDL receptor-related protein) are known to bind apoE specifically. Using LDL-receptor negative cells and LRP-deficient cell mutants, as well as HS-deficient cells, we have found that the heparin-mimicking anionic compounds bind preferentially to the LDL receptor-related protein (LRP). This binding appears to exert an inhibitory signal, resulting in inhibition of vascular SMC proliferation.

METHODS

Reagents

Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g glucose/L), Ham's F-12 Medium, calf serum, fetal calf serum, penicillin, streptomycin, and saline containing 0.05% trypsin, 0.01 M sodium phosphate, and 0.02% EDTA (STV) were obtained from Biological Industries (Beit-Haemek, Israel). Sodium heparin from porcine intestinal mucosa (Mr 14,000, antifactor Xa 165 IU/mg) was obtained from Hepar Industries (Franklin, OH). Lactoferrin and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). Bacterial (*Flavobac-*

terium heparinum) heparinase II was kindly provided by Dr. J. Zimmermann (IBEX Technologies, Montreal, Canada). $Na^{125}I$ and ^{125}I-labeled Bolton–Hunter reagent was from Amersham (Buckinghamshire, England). Lumax scintillation fluid was from Lumac System Inc. (Titusville, FL). Recombinant human apolipoprotein E3 (apo E) was produced by BioTechnology General (Rehovot, Israel) as described [Vogel et al., 1985]. Compound RG-13577 (polymer of 4-hydroxyphenoxyacetic acid and formaldehyde ammonium salt, Mr~5,800) and ^{14}C-labeled RG-13577 (25.6 mCi/mg) were synthesized and kindly provided by Dr. John Regan and Dr. Michael Chang, Rhone-Poulenc Rorer Co. (Collegeville, PA) [Regan, et al., 1993]. Computer-assisted molecular modeling suggests that compound RG-13577 forms a super structure which ensures a regular spatial distribution of negative charges, similar to that of heparin. Tissue culture dishes were from Falcon Labware Division, Becton Dickinson & Co. (Oxnard, CA) and 24-well tissue culture plates were obtained from Nunc (Roskilde, Denmark). All other chemicals were of reagent grade and were purchased from Sigma.

Cells

SMCs were isolated from the bovine aortic media as previously described [Castellot et al., 1986]. Briefly, the abdominal segment of the aorta was removed and the fascia cleaned away under a dissecting microscope. The aorta was cut longitudinally, and small pieces of the media were carefully stripped from the vessel wall. Two or three such strips with average dimensions of 2×2 mm were placed in 60-mm tissue culture dishes that contained DMEM supplemented with 10% FCS, 100 u/ml penicillin, and 100 μg/ml streptomycin. Within 7–14 days, large patches of multilayered cells had migrated from the explants. Approximately 1 week later, the cells were subcultured into 100-mm tissue culture plates ($4–6 \times 10^5$ cells/plate). The cultures (passage 3–8) exhibited typical morphological characteristics of vascular SMCs and the cells were specifically stained with monoclonal antibodies that selectively recognize muscle form of actin (HHF-35). This antibody does not recognize endothelial cells or fibroblasts [Tsukada et al., 1987; Benezra, et al., 1994].

Wild-type Chinese hamster ovary cells (CHO-KI) were obtained from American Type Culture Collection (Rockville, MD). Glycosaminoglycan-deficient CHO mutant, *pgs*A-745 cells were obtained as previously described [Esko et al., 1985; Lidholt et al., 1992] and kindly provided by Dr. J. D. Esko (Department of Cellular and Molecular Medicine, University of California, San Diego). Mutant *pgsA*-745 cells are deficient in xylosyltransferase which catalyzes the first sugar transfer step in GAG biosynthesis. The total sulfated GAGs produced by these cells is $< 5\%$ of the amount made by the wild-type cells [Esko et al., 1985; Lidholt et al., 1992].

Homozygous (PEA 13) LRP-deficient mouse embryonic fibroblasts were obtained using pseudomonas exotoxin A (PEA) as the selection agent [Willnow et al., 1994] and were kindly provided by Dr. Joachim Herz (Department of Molecular Genetics, University of Texas, Southwestern Medical Center, Dallas, TX). Briefly, mouse embryos were teased apart in 0.05% (w/v) trypsin solution and incubated at 4°C overnight followed by 30 min at 37°C. The softened tissue was disrupted by repeated pipetting and the supernatant was plated in 75 cm^2 tissue culture flasks to obtain primary embryonic fibroblasts [Willnow et al., 1994]. To isolate cell lines in which both LRP alleles are functionally destroyed, primary embryonic fibroblasts were isolated from embryos heterozygous for the LRP disruption and cultured in the presence of G418. The cells were then exposed to 30 ng/ml PEA for 24 h and a pool of toxin-resistant cells was obtained after three successive rounds of selection and replating [Willnow et al., 1994]. The cells were subjected to two rounds of toxin selection, cloned, expanded, and analyzed by Southern blotting. Both the normal and LRP-deficient fibroblasts were cultured in DMEM containing 10% FCS [Willnow et al., 1994]. Normal human skin fibroblasts (NSF) and skin fibroblasts (S-233) from an LDL receptor-negative homozygous familial hypercholesterolemic patient were obtained and cultured in DMEM containing 10% FCS, as described [Eisenberg et al., 1992]

Iodination of apoE. Recombinant apoE (100 μg) was incubated (2.5 h on ice) with 0.5 μCi of ^{125}I-labeled Bolton–Hunter reagent in 30 μl of 100 mM sodium borate buffer, pH 8.0. Excess Bolton–Hunter reagent was quenched by adding 200 μl of 0.2 M glycine and incubating for 45 min on ice [Raja et al.,

1984]. The labeled apoE was subjected to gel filtration on a Sephadex G25 column (PD 10, Pharmacia) and kept for up to 3 weeks at 20°C in 2 mM sodium bicarbonate buffer (pH 8.0) containing 1 mM cysteine and 0.2% BSA. The specific activity of the ^{125}I-apoE was 1.2×10^5 cpm/μg.

Iodination of LDL. LDL (d 1.019–1.063 g/ml) was separated from plasma obtained from normal human subjects as previously described [Eisenberg et al., 1992]. ^{125}I-labeled LDL was prepared by the iodine monochloride method as modified by Bilheimer et al. [1972]. The iodinated preparation was dialyzed extensively against 0.15 M NaCl, 0.001% EDTA, pH 7.4 and the final specific activity varied between 50–500 cpm/ng protein. In all preparations $< 95\%$ of the radioactivity was precipitated by TCA and $< 5\%$ was extractable by chloroform-methanol.

Preparation of lipid emulsions. Model intermediate density lipoprotein (IDL)-sized emulsions were prepared as previously detailed [Schweigelshohn et al., 1995] by combining an equal weight of triolein and cholesteryl oleate with twice the amount of egg yolk phosphatidylcholine. To measure the uptake of triglyceride particles (TGRP), [^{3}H]-cholesteryl hexadecyl ether, [cholesteryl-1,2-^{3}H (N)] was added in the ratio of 2 μCi/mg core lipid. Because [^{3}H]- cholesteryl ether is not metabolized by mammalian cells, it can be used as a non-degradable marker for particle internalization by cells.

Preparation of apoE-lipid emulsion complexes. For experiments with apoE, emulsions were incubated with apoE (with gentle agitation every 5 min) at room temperature for 30 min, which allows sufficient time for equilibrium binding [Tajima et al., 1983; Oswald et al., 1987].

Binding of ^{14}C-RG-13577. Cells were grown to confluency in 16-mm culture wells ($4–5 \times 10^5$ cells/well). Confluent cultures were transferred to 4°C, washed once with PBS followed by incubation in DMEM containing 0.2% bovine serum albumin (BSA) and 20 mM HEPES. ^{14}C-RG-13577 (1 μg/ml, except when stated otherwise) was then added and the cultures were incubated at 4°C for 2 h, washed ($\times 3$) with DMEM containing 0.2% BSA and dissolved in 0.2 M NaOH. Radioactivity was determined by counting in a Beckman model 5500 γ-counter [Benezra et al., 1994]. Nonspecific binding, determined in the presence of 250–500-fold excess unlabeled RG-13577, did not exceed 20% of the total binding.

Binding of ^{125}I-apoE. Cells were grown to confluency in 16-mm culture wells ($4–5 \times 10^5$ cells/well). Confluent cultures were transferred to 4°C, washed once with PBS followed by incubation in PBS containing 0.01% $CaCl_2$, 0.01% $MgCl_2$ and 0.2% BSA. ^{125}I-apoE was then added at the indicated concentrations and the cultures were incubated at 4°C for 2 h, washed ($\times 3$) with DMEM containing 0.2% BSA and dissolved in 1 M NaOH. Radioactivity was determined by counting in a γ-counter.

Binding of ^{125}I-LDL. Cells were grown in 16-mm culture wells in DMEM containing 10% FCS. On the seventh day after seeding, monolayers were washed with PBS and incubated for 48 h in DMEM containing 5% lipoprotein-deficient serum (LPDS) prepared as described [Eisenberg et al., 1992]. The cells were then incubated (4°C, 2 h) with DMEM containing 5% BSA and 5 μg/ml ^{125}I-LDL and washed three times with DMEM containing 0.2% BSA. The cell layer was dissolved in 0.5 ml 1 M NaOH and the cell-associated radioactivity counted in a γ-counter. Nonspecific binding determined in the presence of 150-fold excess unlabeled LDL did not exceed 20% of the total binding.

RESULTS

Competitive Binding of ^{14}C-RG-13577 and ^{125}I-apoE to SMCs

Migration and proliferation of vascular SMCs could be inhibited by heparin and heparan sulfate in pathological processes such as arteriosclerosis and restenosis [Castellot et al., 1986; Schmidt et al., 1992]. Likewise, "heparin mimicking" compounds such as polyhydroxyphenoxy acetic acid (RG-13577) were found to inhibit the proliferation of vascular SMCs [Benezra et al., 1994]. These polyanionic molecules exhibited specific and saturable binding to SMCs, interacting with a high molecular weight cell surface protein as revealed by cross-linking experiments [Benezra et al., 1994]. The

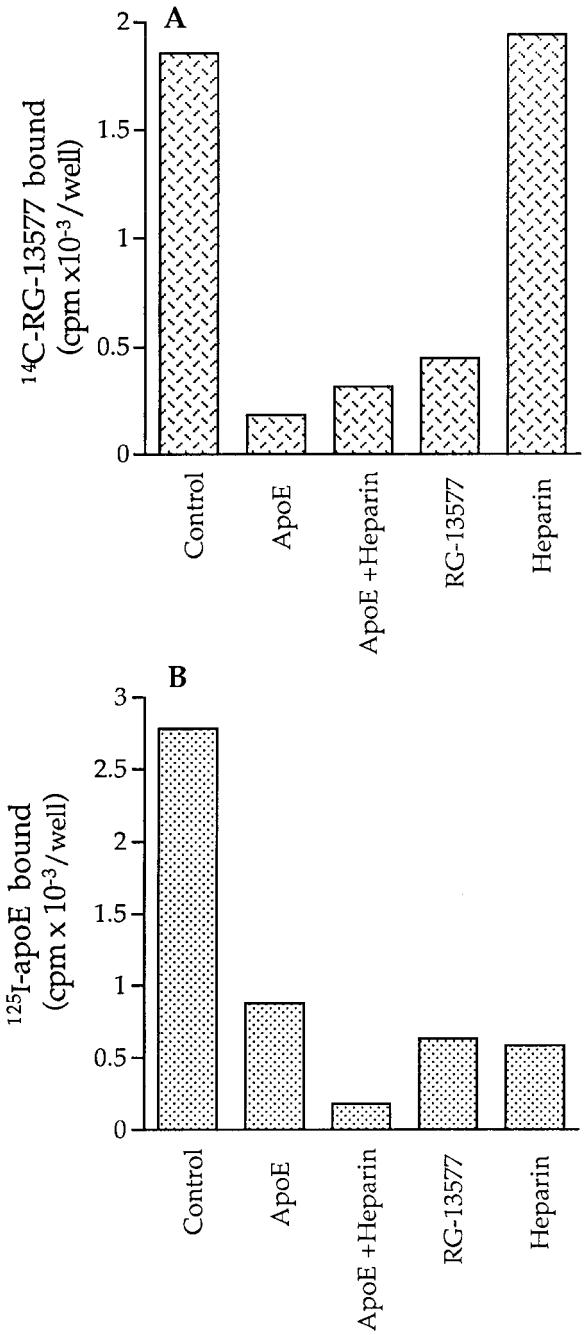


Fig. 1. Binding of RG-13577 and apoE to vascular SMCs. Confluent cultures of SMCs (24-well plates) were incubated (2 h, 4°C) with **A**. ^{14}C-RG-13577 (1 μg/ml), or **B**. ^{125}I-apoE (1.6 μg/ml) in the absence (control) or presence of excess unlabeled apoE (100-fold), RG-13577 (250-fold) or heparin (250-fold). ^{14}C-RG-13577 was also incubated with SMCs in the presence of apoE (1 mg/ml) that was first incubated (30 min, 24°C) with heparin (2.5 μg/ml) (apoE+heparin). At the end of the incubation, the cells were washed (× 3), solubilized with NaOH and cell-associated radioactivity counted in a γ-counter. Each data point is the mean of triplicate wells and the standard deviation did not exceed ± 15%. At least 3 independent experiments were performed. A representative experiment is presented in Figure 1 and the variation between different experiments did not exceed ± 20%.

binding of RG-13577 was efficiently competed by an excess of non-radioactive RG-13577, as well as by apolipoprotein E (apoE), but not by an excess of heparin (Fig. 1A).

Since apoE binds to heparin and HS, its efficient competition with RG-13577 binding to cells may be due to interaction, in solution, of RG-13577 with the heparin binding domain of apoE. To exclude this possibility, apoE was first incubated with excess heparin, followed by incubation with SMCs in the presence of heparin and ^{14}C-RG-13577. Similar results were obtained with or without preincubation of apoE with heparin (Fig. 1A). To further investigate whether the effects of RG-13577 are the result of a direct interaction with apoE, additional experiments were performed under conditions where apoE-to-RG-13577 ratios were either very high or very low (data not shown). First, ^{14}C-RG-13577 (2 μg) (MW = 5800) was incubated for 30 min with 20 μg apoE (MW = 38.000 kD) complexed to IDL-size model lipid emulsion (200 μg neutral lipid/ml) and the mixture was centrifuged in a SW41 rotor for 60 min, conditions that result in flotation of the apoE–lipid complex. Distribution of ^{14}C-RG-13577 was uniform throughout the centrifuge tube, and was not different from tubes containing model emulsions without apoE, or from tubes containing ^{14}C-RG-13577 alone. Second, in another experiment, apoE-emulsions were eluted over Sepharose CL2B columns, in the absence or presence of RG-13577 (50 μg RG-13577 to 300 μg emulsion with 30 μg apoE). Addition of RG-13577 did not result in any displacement of apoE from particles as assayed by dot blot. These results (not shown) indicate that the effects of RG-13577 to be described below are not due to direct interaction between RG-13577 and apoE.

Subsequent experiments were undertaken to determine whether apoE shares the same binding specificity as compound RG-13577. For this purpose, SMCs were incubated with ^{125}I-apoE in the absence or presence of excess apoE, apoE plus heparin, RG-13577, or heparin. As demonstrated in Figure 1B, binding of ^{125}I-apoE was efficiently competed by both compound RG-13577 and heparin. It therefore appears that compound RG-13577 and apoE bind to a common receptor, but differ in their ability to interact with heparan sulfate and/or heparin-like domains on the SMC surface.

Effect of Heparinase on Binding of RG-13577 and apoE to SMCs

The involvement of the cell surface heparan sulfate in apoE and RG-13577 binding to SMCs was further investigated. Confluent cultures of SMCs were treated (2 h, 37°C) with increasing concentrations of heparinase II (0.05–1.25 IU/ml), a bacterial enzyme capable of degrading both low sulfate and high sulfate regions in heparan sulfate [Lolse and Linhardt, 1992]. Studies performed with sulfate ($Na_2{}^{35}SO_4$)-labeled SMCs revealed that more than 95% of the cell surface heparan sulfate was degraded in cells exposed to as little as 0.1 IU/ml heparinase as revealed by release of labeled HS degradation fragments from the cells (not shown). Heparinase-treated SMCs also failed to bind ^{125}I-bFGF to low affinity cell surface heparan sulfate binding sites [Eisenberg et al., 1992]. Heparinase-treated SMCs were incubated (2 h, 4°C) with ^{14}C-RG-13577 or ^{125}I-apoE in the absence or presence of excess unlabeled RG-13577 or apoE, respectively. As shown in Figure 2A, pretreatment with heparinase failed to reduce specific binding of ^{14}C-RG-13577 to SMCs. In contrast, binding of ^{125}I-apoE was reduced by about 60% in cells treated with 0.05–0.1 IU heparinase II (Fig. 2B). Pretreatment with chondroitinase ABC had no effect on the binding of either ^{14}C-RG-13577 or ^{125}I-apoE to SMCs (not shown). Addition of excess (~250-fold) unlabeled chondroitin sulfate, dermatan sulfate, hyaluronic acid, laminin or fibronectin did not affect the binding of ^{14}C–RG-13577 or ^{125}I-apoE to the SMC surface (not shown). These experiments indicate that heparan sulfate does not participate in the binding of RG-13577 to the SMCs, as opposed to its involvement in cell surface binding of apoE.

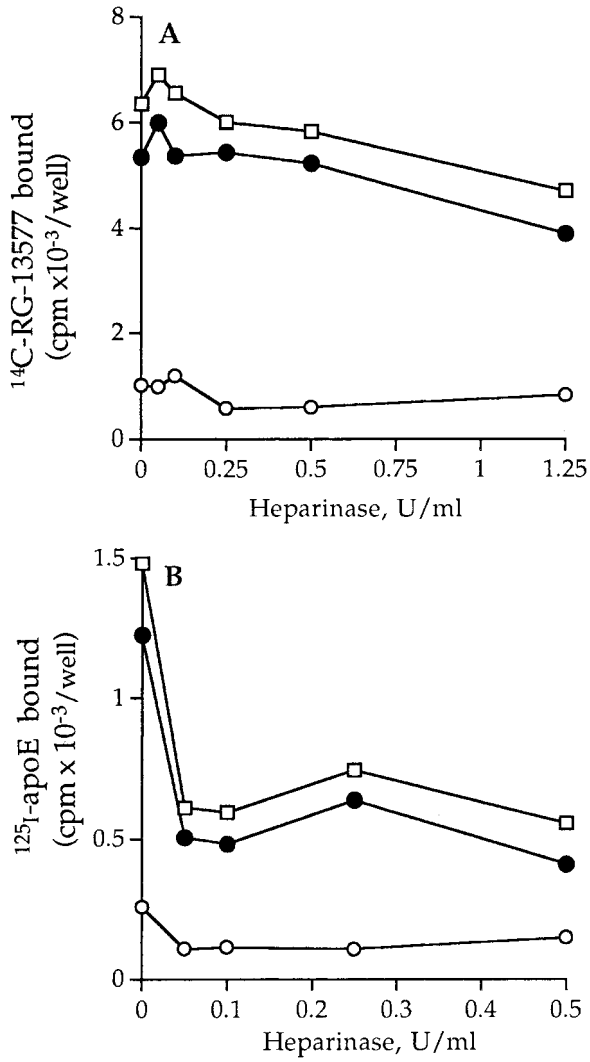


Fig. 2. Effect of heparinase on binding of RG-13577 and apoE to vascular SMCs. Confluent cultures of SMCs were pretreated (2 h, 37°C, binding medium) with increasing amounts of bacterial heparinase II. The cells were then incubated (2 h, 4°C) with (**A**) ^{14}C-RG-13577 (1 μg/ml), or (**B**) ^{125}I-apoE (2 μg/ml), in the absence (□) or presence (○) of 250-fold excess unlabeled RG-13577 (A), or apoE (B). Specific binding (●) was obtained by subtracting the nonspecific binding (○) from the total binding (□). The variation between triplicate determinations did not exceed ± 10% of the mean.

Binding of RG-13577 and apoE to Wild-Type and HS-Deficient Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cells have abundant heparan sulfate proteoglycans on their cell surface. The CHO mutant *pgsA*-745 does not express heparan sulfate due to a deficiency in xylosyltransferase which is required to initiate glycosaminoglycan synthesis onto the proteoglycan core protein [Esko et al., 1985; Lidholt et al., 1992]. These cell mutants were utilized to further demonstrate that heparan sulfate is not involved in RG-13577 cell surface binding. Confluent cultures of wild-type or *pgs*A-745 CHO cells were incubated (2 h, 4°C) with 1 μg/ml ^{14}C-RG-13577 in the absence and presence of increasing concentrations of unlabeled RG-13577 or apoE. Binding of ^{14}C-RG-13577 to wild-type and *pgs*A-745 cells was similar and was equally displaced by excess unlabeled RG-13577 (Fig. 3) and apoE (not shown), regardless of whether HS was

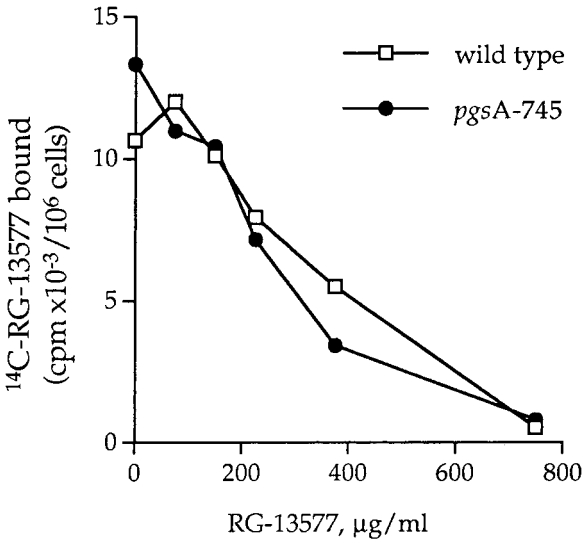


Fig. 3. Binding of RG-13577 to wild-type (KI) and heparan sulfate deficient (*pgs* A-745) CHO cells. Confluent cultures of KI (□) and *pgs* A-745 (●) CHO cells were incubated (2 h, 4°C) with ^{14}C-RG 13577 (1 μg/ml), in the absence and presence of increasing concentrations of excess RG-13577. At the end of the incubation, the cells were washed (×3), solubilized with NaOH and cell-associated radioactivity was counted in a γ-counter. Each data point is the mean of triplicate wells and the standard deviation did not exceed ± 15%.

expressed or not on the cell surface. These results further indicate that the cell surface HS does not contribute to RG-13577 binding. On the other hand, binding of ^{125}I-apoE to wild-type CHO cells was two-fold higher as compared to the HS-deficient *pgs*A-745 mutant cells. This binding was displaced by both excess unlabeled RG-13577 or apoE (not shown). Altogether these results suggest that RG-13577 and apoE may share another common cell surface receptor, possibly the LDL receptor or the LDL receptor-related protein (LRP), the two other known cell surface receptors for apoE [Willnow et al., 1994, 1999; Herz et al., 2000].

Binding of ^{14}C-RG-13577 and ^{125}I-apoE to LDL Receptor Negative Fibroblasts

The following experiments were undertaken to elucidate whether compound RG-13577 may bind to the LDL receptor in a manner similar to apoE. For this purpose, ^{14}C-RG-13577, ^{125}I-apoE or ^{125}I-LDL were incubated with normal human skin fibroblasts (NSF) and with LDL receptor negative fibroblasts (S-233) derived from a homozygous familial hypercholesterolemic patient [Friedman et al., 1990; Sehayek et al., 1991]. As expected, there was little or no specific binding of both ^{125}I-LDL (Fig. 4A), or ^{125}I-apoE (Fig. 4B) to the LDL receptor negative (S-233) cells, as compared to normal human skin fibroblasts (NSF). On the other hand, the LDL receptor positive (NSF) and negative (S-233) fibroblasts bind ^{14}C-RG-13577 to a similar extent (Fig. 4C). This binding was fully competed by an excess of unlabeled RG-13577 or apoE, but there was almost no effect on excess heparin or LDL (Fig. 4C). In contrast, cell surface binding of ^{125}I-apoE was efficiently competed in the presence of either RG-13577, apoE, heparin, or LDL (Fig. 4B). Binding of ^{125}I-LDL to normal skin fibroblasts was displaced by excess apoE, heparin, or LDL, but not by an excess unlabeled compound RG-13577 (Fig. 4A). These experiments (Fig. 4) further indicate that compound RG-13577 does not bind to the LDL receptor.

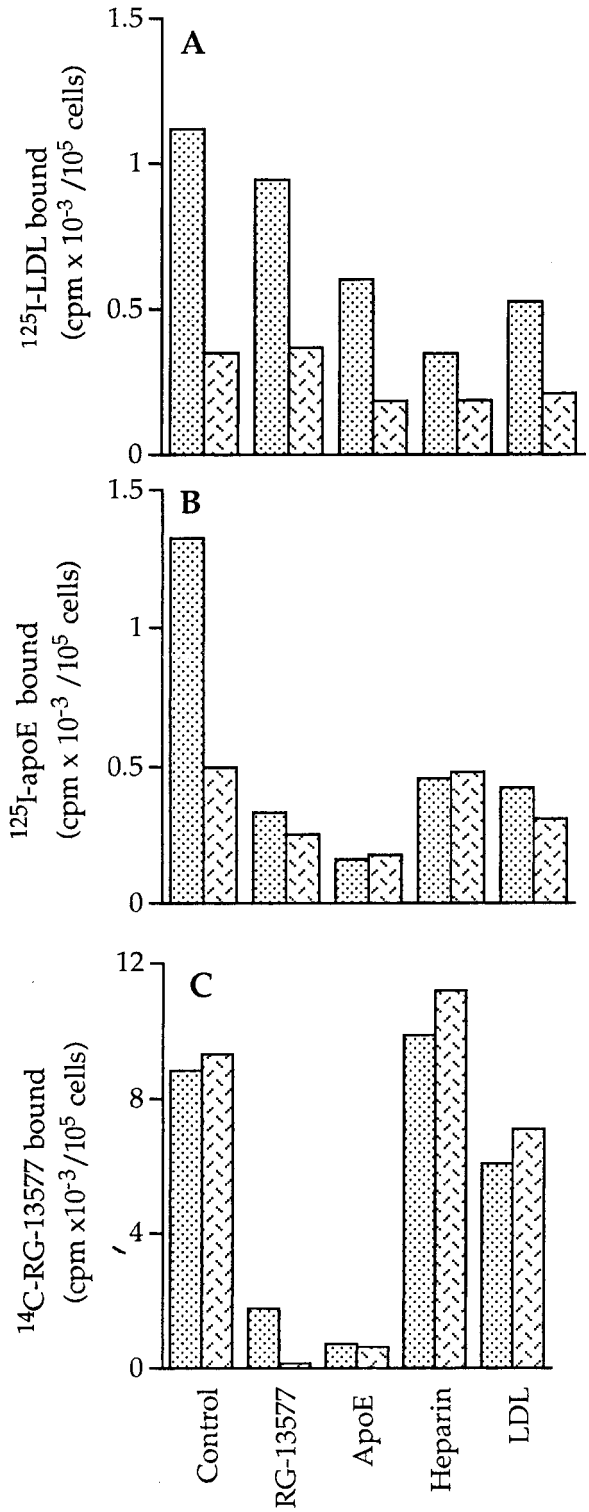
In other experiments, apoE emulsions were incubated with normal human skin fibroblasts under conditions where almost all particle uptake is via the LDL receptor, and not via LRP [Al-Haideri et al., 1997]. No differences were observed in apoE-emulsion particle uptake in the absence and presence of RG-13577 (50 μg/ml). This result indicates that RG-13577 does not affect the ability of apoE to bind to the LDL receptor.

Interaction of Compound RG-13577 With the LDL Receptor-Related Protein (LRP)

Effect of lactoferrin. Lactoferrin, a 70 kDa iron-binding glycoprotein, was reported to inhibit the hepatic uptake of chylomicron remnants by binding specifically to the LDL receptor-related protein (LRP) [Gerstein et al., 1993]. Lactoferrin is therefore regarded as one of the ligands that specifically bind to LRP. In order to further identify the receptor site for RG-13577 and related compounds, we investigated whether lactoferrin competes with the binding of ^{14}C-RG-13577 to SMCs. For this purpose, confluent cultures of SMCs were incubated (2 h, 4°C) with ^{14}C-RG-13577 in the presence of increasing concentrations (up to 625 μg/ml) of RG-13577, or lactoferrin (Fig. 5A). Lactoferrin was found to compete well with RG-13577 binding, yielding a 75% reduction in binding at 125 μg/ml lactoferrin. Likewise,

lactoferrin efficiently competed with apoE binding to SMC (Fig. 5B).

These results were further corroborated by showing that lactoferrin can protect vascular SMCs from the inhibitory effect of compound RG-13577. As demonstrated in Figure 6A, SMC proliferation was inhibited by about 80% in the presence of 5 μg/ml RG-13577. This inhibition was much smaller in the presence of 125 μg/ml lactoferrin, as indicated by a 2–3-fold increase in cell number and by restoration of the normal SMC morphological appearance (Fig. 6B) in the presence of both lactoferrin and RG-13577, as compared to cells incubated with compound RG-13577 alone.



Effect of the 39 kDa LRP associated protein (RAP). SMCs were incubated with ^{14}C-RG-13577 in the absence or presence of a 39 kDa protein (LRP associated protein-RAP) which copurifies with LRP and regulates the binding of other ligands to LRP [Orlando and Farquhar, 1994; Zheng et al., 1994; Bu, 1998]. As demonstrated in Figure 7, SMC binding of RG-13577 was reduced by 70–80% in the presence of 250-fold excess unlabeled RAP (Fig. 7). The above results with lactoferrin and RAP suggest that compound RG-13577 and related polyanionic aromatic compounds are capable of specific interaction with the LRP.

LRP-deficient cell mutants. In a subsequent experiment we utilized murine embryonic fibroblasts that are genetically deficient of LRP [Willnow et al., 1994]. The ability of homozygous LRP-deficient mouse embryonic fibroblasts (PEA-13) [Willnow et al., 1994] to bind ^{14}C-RG-13577 or ^{125}I-apoE was compared to wild-type embryonic mouse fibroblasts (EMF). Incubation (24 h, 4°C) of EMF cells with ^{14}C-RG-13577 resulted in specific binding of RG-13577 to the cell layer (Fig. 8). A similar incubation of PEA-13 cells with labeled RG-13577 revealed a 60–70% reduction in binding (Fig. 8) Excess apoE, RG-13577 or lactoferrin reduced the binding of ^{14}C-RG-13577 by 65–

Fig. 4. Binding of LDL, apoE, and RG-13577 to normal and LDL receptor negative human skin fibroblasts. Confluent cultures of normal human skin fibroblasts (▨) and LDL receptor negative (S-233) human skin fibroblasts (▨) were incubated (2 h, 4°C) with (**A**) ^{125}I-LDL (5 μg/ml); (**B**) ^{125}I-apoE (1.25 μg/ml); or (**C**) ^{14}C-RG-13577 (1 μg/ml), in the absence (control) and presence of excess (250-fold) unlabeled RG-13577, apoE, heparin, or LDL. The cells were then washed (× 3), solubilized with NaOH and the radioactivity counted in a γ-counter. The variation between triplicate determinations did not exceed ± 10% of the mean.

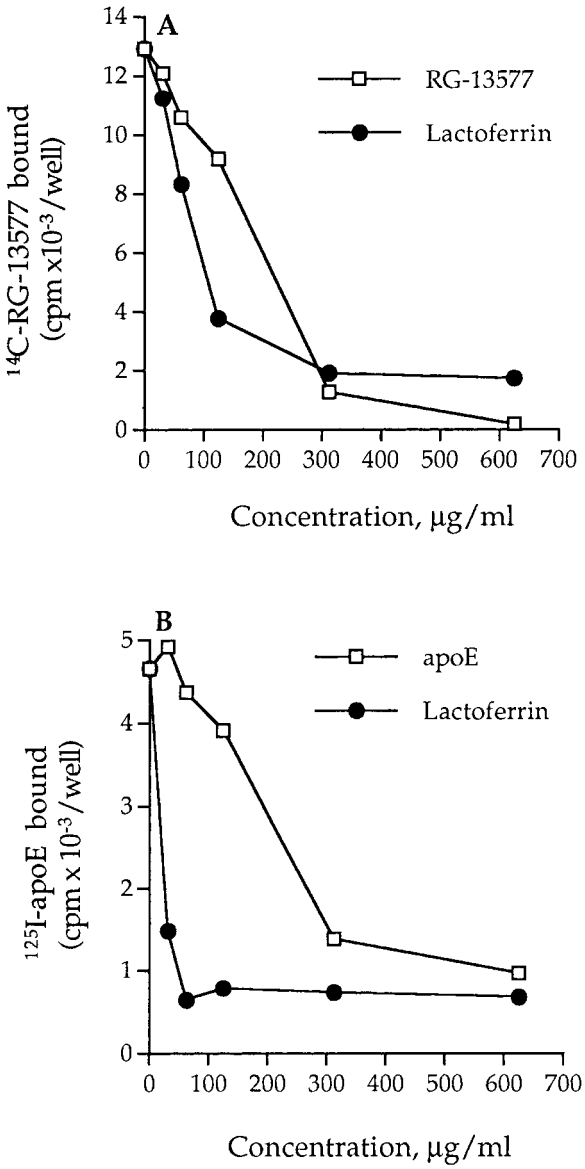


Fig. 5. Effect of lactoferrin on binding of RG-13577 and apoE to SMCs. Confluent cultures of vascular SMCs were incubated (2 h, 4°C) with (**A**) ^{14}C-RG-13577 (1.25 µg/ml), or (**B**) ^{125}I-apoE (1.25 µg/ml), in the absence or presence of increasing concentrations of unlabeled RG-13577 (□), lactoferrin (●), or apoE (□). The cells were washed (× 3) and the amount of cell-associated radioactivity was determined by counting in a γ-counter. A representative experiment is presented in Fig. 1 and the variation between different experiments did not exceed ± 20%.

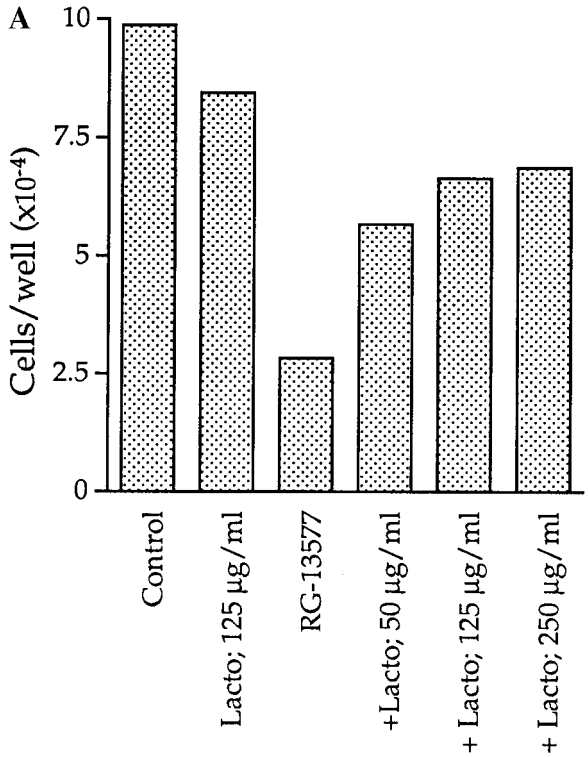


Fig. 6. (**A**) Effect of RG-13577 and lactoferrin on SMC proliferation. SMCs were seeded (5×10^3 cells/16 mm well) in DMEM containing 10% FCS. RG-13577 (5 µg/ml), lactoferrin (125 µg/ml), or RG-13577 plus 50, 125 or 250 µg/ml lactoferrin were added on Day 2 after seeding. The cells were dissociated with STV on day 6 and counted in a Coulter counter. Each data point is the mean of triplicate wells and the SD did not exceed ± 15%.

80%, but there was no effect of heparin (Fig. 8). Binding of ^{125}I-apoE to the LRP-deficient mouse fibroblasts (PEA-13) was reduced by 25% as compared to apoE binding to the wild-type embryonic mouse fibroblasts (not shown).

DISCUSSION

In a previous study we have demonstrated that the polyanionic compound RG-13577 binds to specific receptors on the surface of vascular SMCs and inhibits their proliferative response to serum, bFGF and thrombin [Benezra et al., 1994]. We now present evidence that the heparin-mimicking compound RG-13577 (polymer of 4-hydroxyphenoxy acetic acid) binds to the LDL receptor-related protein (LRP) on the SMC surface. The LRP is a large multi-functional receptor implicated in the cellular uptake of functionally diverse ligands including α_2-macroglobulin, apoE-enriched β-very low density lipoprotein (β-VLDL), lipoprotein lipase, t-PA/PAI-1 complexes, lactoferrin, pseudomonas exotoxin A, and the 39 kDa LRP-associated protein (RAP) [Brown et al., 1991; Willnow et al., 1994; Zheng et al., 1994]. Binding studies performed with SMCs and labeled RG-13577 or

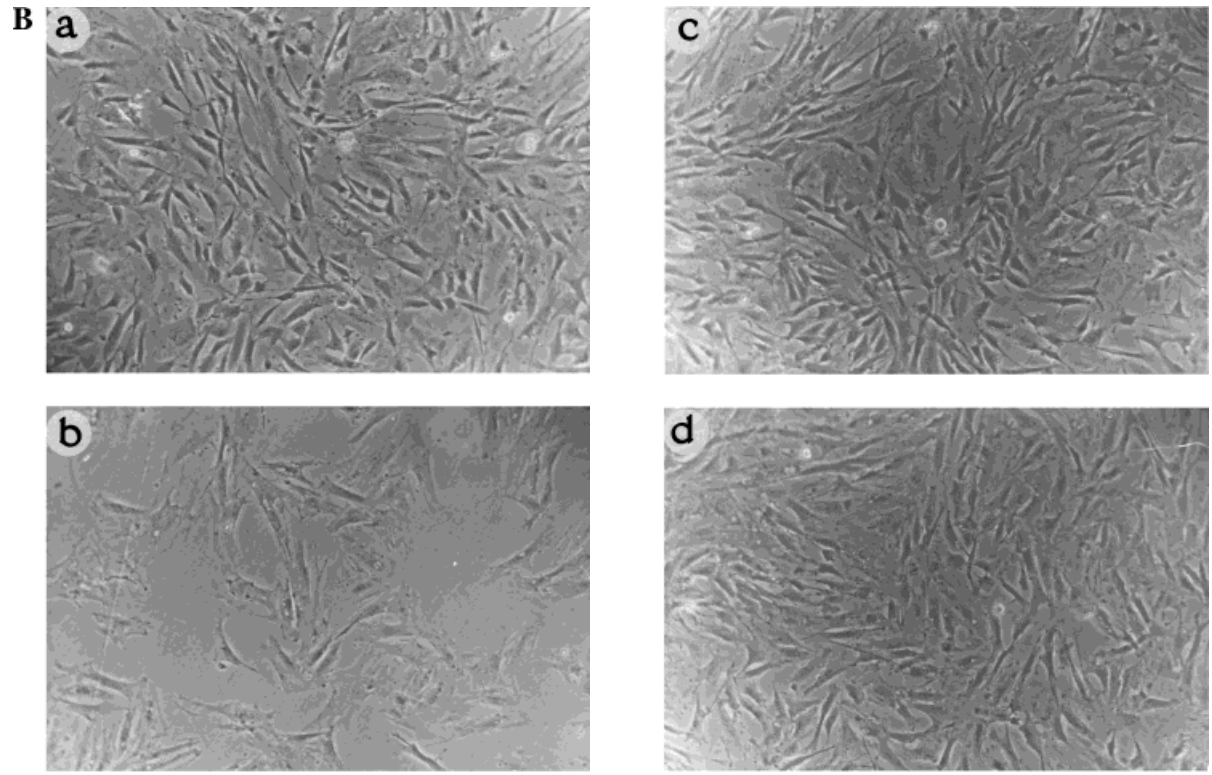


Fig. 6. Effect of RG-13577 and lactoferrin on SMC. (**B**) Morphological appearance. (**a**) Control untreated SMCs; (**b**) SMC treated with 5 μg/ml RG-13577; (**c**) SMC treated with 125 μg/ml lactoferrin; and (**d**) SMC treated with 5 μg/ml RG-13577 and 125 mg/ml lactoferrin. Cells were seeded and treated as described in Figure 6A and phase micrographs (× 200) were taken 6 days after seeding.

apoE revealed that while excess apoE inhibited the binding of both apoE and RG-13577 to SMCs, excess heparin reduced the binding of labeled apoE to SMCs, but failed to compete with SMC binding of RG-13577. Accordingly, treatment of SMCs with bacterial heparinase resulted in a remarkable decrease in the binding of labeled apoE to SMCs, but had no effect on receptor binding of labeled RG-13577. These results indicate that unlike apoE receptor interaction, heparan sulfate proteoglycans (HSPG) do not play a significant role in the binding of compound RG-13577 and related molecules to SMCs. This conclusion was strengthened by studies performed with CHO mutant cells (*pgs*A-745) defective in proteoglycan biosynthesis [Esko et al., 1985]. Specific binding of RG-13577 to these mutant cells was similar to that observed with the wild-type CHO cells. On the other hand, binding of labeled apoE to the same mutant cells was reduced by about 50% as compared to the wild-type cells. It was previously reported that both the *pgs*A-745 GAG deficient cell mutants and the wild-type CHO cells possess a similar amount of LRP as determined by ligand blot analysis and α_2-macroglobulin binding capacity [Zhong-Sheng et al., 1993]. These cell types also possess a similar number of LDL receptor sites, as determined by LDL binding studies [Zhong-Sheng et al., 1993]. These results indicate that HSPG is not involved in binding of RG-13577 to the SMC surface.

Binding experiments performed with LDL receptor negative familial hypercholesterolemic (FH) fibroblasts revealed a 60–80% reduction in both LDL and apoE binding to FH fibroblasts as compared to normal human skin fibroblasts. There was, however, no difference in binding of labeled RG-13577 to both cell types. It was also found that excess unlabeled RG-13577 did not compete for LDL binding, and excess unlabeled LDL did not compete for RG-13577 binding to normal human fibrobalsts. Also, RG-13577 did not interfere with apoE-emulsion uptake in normal fibroblasts.

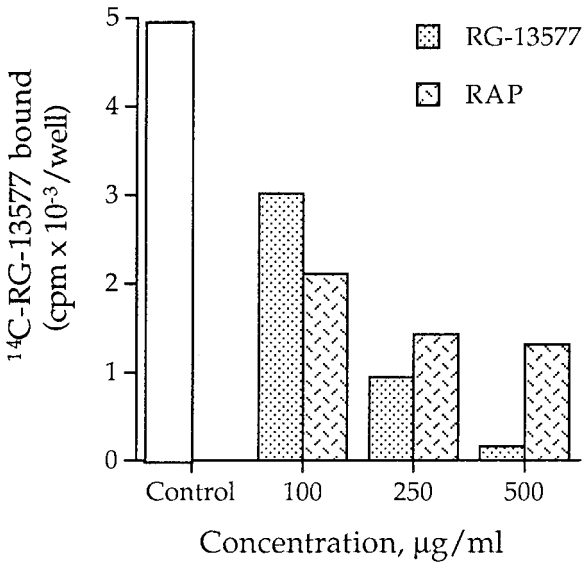


Fig. 7. Effect of the 39 kDa LRP associated protein on binding of RG-13577 to vascular SMCs. Confluent cultures of SMCs were incubated (2 h, 4°C) with ^{14}C-RG-13577 (1.25 μg/ml) in the absence or presence of increasing concentrations of unlabeled RG-13577 (▨) or the 39 kDa LRP associated protein (▨). The cells were washed (× 3), solubilized with NaOH and cell-associated radioactivity counted in a γ-counter. The variation between triplicate determinations did not exceed ± 10% of the mean.

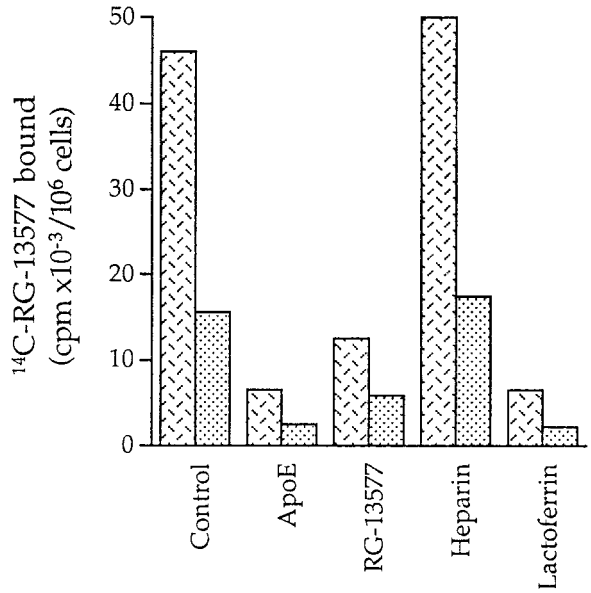


Fig. 8. Binding of RG-13577 to normal and LRP-deficient embryonic mouse fibroblasts. Confluent cultures of normal embryonic mouse fibroblasts (EMF) (▨), or homozygous (PEA-13) (▨) LRP-deficient mouse embryonic fibroblasts were incubated (2 h, 4°C) with ^{14}C-RG 13577 (1 μg/ml), in the absence (control) or presence of excess (250-fold) unlabeled apoE, RG-13577, heparin or lactoferrin. The cell monolayers were washed (× 3), solubilized and subjected to γ counting. The variation between triplicate determinations did not exceed ± 10% of the mean.

These results further indicate that the LDL receptor is not involved in cell surface binding of RG-13577.

The normal binding of RG-13577 to cells that lack the LDL receptors, as well as to cells that lack heparan sulfate on their cell surfaces, together with the efficient displacement of RG-13577 by apoE, suggest that RG-13577 and related compounds may interact with SMCs through binding to LRP cell surface receptor sites. This conclusion was further supported by the inhibitory effect of the 39 kDa LRP associated protein (RAP) which copurifies with LRP and inhibits the binding of other ligands to this receptor [Orlando and Farquhar, 1994; Warshawsky et al., 1994]. The efficient inhibition of RG-13577 binding to SMCs in the presence of apoE, lactoferrin or RAP, may be due in part to a possible interaction, in solution, of each of these heparin-binding proteins with the polyanionic compound RG-13577. Preicubation of apoE, lactoferrin or RAP with heparin prior to their incubation with SMCs in the presence of labeled RG-13577 had no effect on the ability of these proteins to inhibit subsequent cell binding of compound RG-13577. In other studies, preincubation of SMCs with lactoferrin followed by the removal of the unbound protein, resulted in the inhibition of RG-13577 binding, again suggesting no interaction between compound RG-13577 and the cell-associated lactoferrin (not shown). Finally, our experiments with apoE–lipid emulsions indicated that there was no direct interaction between RG-13577 and apoE. In light of these results it appears that RG-13577 interferes with apoE cell binding by interacting with sites other than apoE itself or the LDL receptor.

It was previously demonstrated that lactoferrin binds directly to LRP and blocks the LRP-mediated stimulation of cholesteryl ester synthesis in cultured human fibroblasts elicited by apoprotein–βVLDL, or lipoprotein lipase–βVLDL complexes [Willnow et al., 1992]. In the present study, bovine lactoferrin was found to be a potent inhibitor of RG-13577 binding to SMCs, thus further supporting its possible interaction with LRP on the SMC surface. Moreover, we have demonstrated that addition of lactoferrin protects the SMCs

against the antiproliferative effect of RG-13577, indicating that this inhibition is in fact initiated by RG-13577 binding to LRP receptor sites on the SMC surface. Since lactoferrin binds to glycosaminoglycans and sulfated polysaccharides [Mann et al., 1994; Wu et al., 1995], its protective effect may be due in part to a direct interaction of lactoferrin with HSPGs on the cell surface and possibly also with RG-13577. LRP-deficient embryonic mouse fibroblasts were obtained using pseudomonas exotoxin A as a selection agent [Willnow et al., 1994]. These mutant cells were unable to bind, internalize, and degrade methylamine-activated α_2-macroglobulin, t-PA/PAI-1 complexes, as well as the LRP receptor associated 39 kDa accessory protein [Willnow et al., 1994]. Our studies on the binding of labeled RG-13577 to homozygous (PEA-13) LRP-deficient embryonic fibroblasts, revealed a marked, albeit incomplete reduction in RG-13577 binding as compared to wild-type embryonic mouse fibroblasts. Cellular binding of ^{14}C-RG-13577 in the presence of excess unlabeled RG-13577, apoE, lactoferrin or RAP ranged from 10 to 25% in different experiments. This may be due, in part, to nonspecific stickiness of the compound to cell surface constituents and the tissue culture plastic. Specific interaction of ^{14}C-RG-13577 with cell surface molecules other than LRP cannot be excluded, as indicated by some specific binding of RG-13577 to LRP-deficient mouse fibroblasts. The presence of heparin-binding sites in growth-arrested vascular SMCs was previously reported [Castellot et al., 1985], although the nature of these receptors was not elucidated.

The extracellular domain of LRP consists of four separate regions (I, II, III, IV), each of which is characterized by LDL receptor ligand-binding type repeats which are followed by one or more epidermal growth factor (EGF) precursor homologous [Brown et al., 1991]. It was shown that the various LRP ligands bind to different sites on the LRP receptor. For example region II was found to be responsible for endocytosis and degradation of t-PA/PAI-1 complexes, but not for uptake of α_2-macroglubulin. The region in LRP involved in its specific interaction with RG-13577 and related compounds has not been identified.

ApoE binds to cell surface heparan sulfate, LDL receptor, and the LRP [Zhong-Sheng et al., 1993]. It has been suggested that heparan sulfate mediates cell surface binding of apoE-enriched remmants, followed by subsequent participation of the LRP in the uptake of the heparan sulfate-bound lipoproteins [Beisiegel et al., 1991]. Such a dual receptor mechanism was demonstrated in studies with members of the heparin-binding family of growth factors (i.e., bFGF, aFGF, VEGF, HB-FGF) [Yayon et al., 1991; Gitay-Goren et al., 1992]. Interaction of heparin with the heparin-binding domain in apoE may interfere with its ability to interact with the LRP, as revealed by an almost complete inhibition of apoE binding in the presence of excess heparin. Unlike this situation, it appears that the binding of RG-13577 involves direct interaction with the LRP and hence is not affected by heparin/ heparan sulfate. Although the natural growth inhibitory ligand that binds specifically to LRP has not yet been identified, it is conceivable that the antiproliferative activity of compound RG-13577 is initiated by such binding. Receptor activation may then elicit an antiproliferative signal through modulation of tyrosine phosphorylation [Miao et al., 1997], kinase C activity [Schmidt et al., 1999], or other cell cycle-related activities, as suggested for other polyanionic compounds such as suramin [Sartor et al., 1992] and heparin [Wright et al., 1989].

In summary, we have identified a series of synthetic polyanionic molecules that efficiently inhibit SMC proliferation, possibly through binding to LRP and/or related cell surface receptors. The structural determinants involved in this receptor interaction are being analyzed in an attempt to identify appropriate compound(s) that may affect the progression of atherosclerosis and restenosis.

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