Density- and proliferation status-dependent expression of T-cadherin, a novel lipoprotein-binding glycoprotein: a function in negative regulation of smooth muscle cell growth?

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Abstract The atypical low density lipoprotein (LDL) binding proteins (M_r 105 and 130 kDa; p105 and p130) in human aortic medial membranes and cultured human and rat aortic smooth muscle cells (SMC) have recently been identified as the cell adhesion glycoprotein T-cadherin. Although cadherins are generally recognized to be important regulators of morphogenesis, the function of T-cadherin in the vasculature is poorly understood. This study has examined the relationship between expression of T-cadherin and the density and proliferation status of SMC. T-cadherin (p105 and p130) levels in SMC lysates were measured on Western blots using ligand-binding techniques. T-cadherin expression was dependent upon cell density, and maximal levels were achieved at confluency. T-cadherin levels were reversibly modulated by switching cultures between serum-free (upmodulation) and serum-containing (downmodulation) conditions. Platelet-derived growth factor (PDGF)-BB, epidermal growth factor (EGF) or insulin-like growth factor (IGF) elicited a dose- and time-dependent downmodulation that was reversible after transfer of SMC to growth factor-free medium. Our results support the hypothesis that T-cadherin may function as a negative determinant of cell growth.

Key words: T-cadherin; Low density lipoprotein; Smooth muscle cell

1. Introduction

Human medial tissue and cultured human and rat aortic smooth muscle cells (SMC) contain two atypical lipoprotein-binding proteins with molecular weights of 105 kDa (p105) and 130 kDa (p130) [1,2]. Binding of low-density lipoprotein (LDL) to these proteins is specific and displays properties that are distinct from those of all other previously described lipoprotein recognizing proteins [1,2]. Partial amino acid sequencing of p105 purified from human medial tissue revealed its putative identity as the glycosylphosphoinositol-anchored cell adhesion glycoprotein T-cadherin [3]. Common identity of p105 and p130 as T-cadherin in cultured human and rat aortic SMC was deduced on the basis of their common immunoreactivity with anti-T-cadherin peptide antibodies and susceptibility to phosphatidylinositol-specific phospholipase C cleavage [4]. Additionally, and in accordance with data in chicken T-cadherin transfected Chinese hamster ovary (CHO) cells [5], trypsin treatment of SMC in the presence of calcium results in a synchronous loss of precursor T-cadherin (p130) and increase of the trypsin/calcium-insensitive mature T-cadherin (p105) [4]. Each member of the cadherin family has a unique calcium-dependent homophilic binding specificity which link together cells expressing the same cadherin type [6–9]. These homophilic interactions define the adhesion selectivities important in regulating cell adhesion, morphology and motility [6–9].

In the nervous system developmental studies in chick have suggested that T-cadherin expression might be a negative determinant of synaptogenesis in skeletal muscle and that it may serve as a barrier to motor neuron sprouting [10,11]. A negative function in growth control is supported by in vitro studies of neurite growth on T-cadherin substrata whereby T-cadherin was found to serve a homotypic avoidance function [11]. Further evidence for a negative role for T-cadherin in cell growth derives from the observation that T-cadherin mRNA is either absent or significantly reduced in human breast carcinoma lines and breast cancer specimens [12]. Transfection of a breast carcinoma with T-cadherin cDNA significantly inhibited growth, and this inhibition was overcome by inclusion of anti-T-cadherin antibodies [12]. The role of T-cadherin in the vasculature is unclear. However, some function associated with growth control can be deduced from our recent observations that T-cadherin levels in SMC can be altered by mitogenic (decreased) and anti-mitogenic (increased) compounds [4]. In order to more specifically define the role for T-cadherin in growth control of SMC we conducted a detailed investigation of the density-dependent and proliferation-status dependent expression of T-cadherin protein.

2. Materials and methods

2.1. Preparation of lipoproteins and lipoprotein-deficient serum

Lipoproteins (low-density (LDL), high-density (HDL) and very low-density (VLDL)) were isolated from the plasma of healthy male humans using sequential buoyant density centrifugation techniques [13]. LDL (density 1.019–1.063 g/ml) was biotinylated using D-biotin-*N*-hydroxysuccinimide [14]. Lipoprotein-deficient serum (LPDS) was prepared from fetal calf serum (FCS) [15].

2.2. Smooth muscle cell culture

Procedures for the isolation, phenotypic characterization and propagation of human aortic SMC (isolated using explant or enzymatic

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Abbreviations: LDL, low density lipoprotein; SMC, vascular smooth muscle cell; LPDS, lipoprotein-deficient serum; FCS, fetal calf serum; SF, serum-free; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IGF, insulin-like growth factor; TBS, Tris-buffered saline; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence

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disaggregation methods) and rat aortic SMC (isolated using enzymatic disaggregation) have been detailed previously [4,16]. For the studies herein, SMC at between passages 4 and 18 were used. SMC were normally cultured in Dulbecco's medium containing 5 mM glutamine, 10 mM TES-NaOH, 10 mM HEPES-NaOH (both at pH 7.3), 0.05 mg/ml gentamycin and 10% FCS. Serum-free medium (SF) was minimal essential medium containing Earle's salts and all ingredients as given above with the exception that serum was substituted with 0.1% (w/v) BSA. Cultures were routinely examined by phase contrast microscopy for visual evaluation of cell densities. Details of the different experimental protocols are described in the text and figure legends. Cells were lysed by addition of 1% SDS/1 mM PMSF [4] and protein concentrations determined using the Lowry method. Lysates were stored at −20°C until use. Even under optimal conditions human aortic SMC grow very slowly in culture and it is difficult to markedly influence their proliferation status by serum/factor supplementation or deprivation. In contrast, rat aortic SMC are readily manipulatable between extremes of rapid growth and quiescence and thus lend themselves to exploitation for studies on growth control. This study has thus preferentially utilized rat SMC. Data obtained with human SMC (in Fig. 5) were qualitatively comparable, but quantitatively less striking.

2.3. Ligand-binding and immuno-blotting

All procedures for polyacrylamide gel electrophoresis, electrotransfer onto nitrocellulose (Schleicher and Schüll), ligand-binding and immuno-blotting, and quantitation using Adobe Photoshop and NIH Image computer programmes have been detailed previously [1–4]. Briefly, SDS-polyacrylamide (8%) gel electrophoresis was performed under non-reducing conditions, and for any given experiment identical amounts of protein (10–50 μg) were loaded per slot. TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.4). containing 5% (w/v) delipidated milk (Rapilait milk, Zurich, Switzerland) was used in all preblocking, incubation and washing procedures, with the exception of incubations with horseradish peroxidase (HRP)-conjugates which used TBS without milk. Use of ligand (LDL)-binding techniques to analyze T-cadherin in smooth muscle cells has been previously validated for whole SMC lysates, membrane extracts from aortic medial tissue and purified T-cadherin [3,4]. In this study biotinylated-LDL (at 80 μg/ml) was used as the ligand in combination with streptavidin-HRP-conjugate and the Amersham ECL detection system. Detection images selected for presentation are those illustrating the reversible (Fig. 2) and specific (Fig. 5) modulation of T-cadherin proteins (p105 and p130). For expression of apo B,E receptor, Gsα, and myosin heavy chain, blots were incubated with monoclonal apo B,E receptor antibodies (Ab-1, Clone 15C8, Calbiochem; recognizing only human and bovine apo B,E receptor), polyclonal anti-Gsα antibodies (K-20, Santa Cruz Biotechnology) or monoclonal anti-myosin (smooth) heavy chain antibodies (Sigma Chemical), and then with the appropriate anti-mouse or anti-rabbit IgG-HRP conjugate.

3. Results and discussion

3.1. Density-dependent expression of T-cadherin protein

We first aimed at determining a relationship between cell density and T-cadherin protein (p105 and p130 forms) expression. Cultures of SMC were enzymatically disassociated using 0.25% trypsin/1 mM EDTA (which results in loss of cellular (surface-located) T-cadherin [4,5]), seeded at different densities and thereafter cultured for up to five days in the presence of 10% FCS. For cultures seeded at the lowest density (0.1×10^6 cells/well), levels of p105 and p130 proteins increased throughout during the 5 day culture period (Fig. 1). These cultures only started to achieve confluence (assessed by phase-contrast microscopy) after the 5th day in culture, and it is noteworthy that at this time point p105 and p130 levels were comparable to the maximal levels achieved in the more densely seeded cultures. In the more densely seeded cultures (0.5 and 1.0×10^6 cell/well), levels of p130 and p105 increased only until such times as the cells achieved confluency and proliferation arrest (2 and 1 days, respectively, for cultures seeded at 0.5 and 1.0×10^6 cell/well). Thereafter levels of p105 and p130 in these cultures decreased. These data indicate that expression levels of T-cadherin protein in passaged SMC are indeed dependent upon cell density, but also appear to be strongly influenced by the proliferation status of the cells.

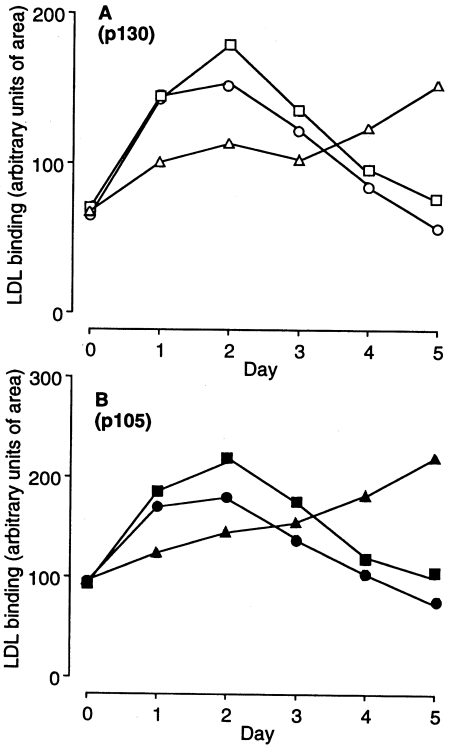

Fig. 1. Dependence of T-cadherin protein levels on cell density. After trypsin/EDTA disaggregation, rat aortic SMC were seeded (day 0) into 6-well dishes at cell densities of $\approx0.1\times10^6$ cells/well (△,▲), $\approx0.5\times10^6$ cells/well (□,■), and $\approx1\times10^6$ cells/well (○,●), and cultured for 5 days in the presence of 10% FCS; confluence in these different cultures was achieved after 5, 2 and 1 days, respectively. Levels of p130 (panel A, open symbols) and p105 (B, closed symbols) in whole cell lysates were measured by ligand-binding as described in Section 2. Two independent experiments were performed and data are given as means ± S.D. of duplicate determinations from each experiment.

3.2. Proliferation status-dependent modulation of T-cadherin protein expression

We next examined the association between T-cadherin protein expression and the proliferation status of SMC. The proliferation status of subconfluent rat aortic SMC was modulated by changing culture conditions. SMC were seeded at a low density and maintained for 24 h under normal growth conditions (i.e. 10% FCS). Cultures were then maintained for 3 days under either serum-containing (Fig. 2A) or serum-deprived conditions (Fig. 2B), and thereafter switched to serum-deprived or serum-containing conditions, respectively, for a further 4 days. For SMC cultured for the first 3 days in the presence of serum increases in p105 and p130 were observed during the first 2 days after which time these cultures attained confluence (Fig. 2A). Cultures initially maintained under SF conditions also exhibited an increase in T-cadherin protein expression during the first 2 days only (Fig. 2B). Although these cultures continued to grow (due to autocrine production of growth factors [17]), albeit only very slowly,

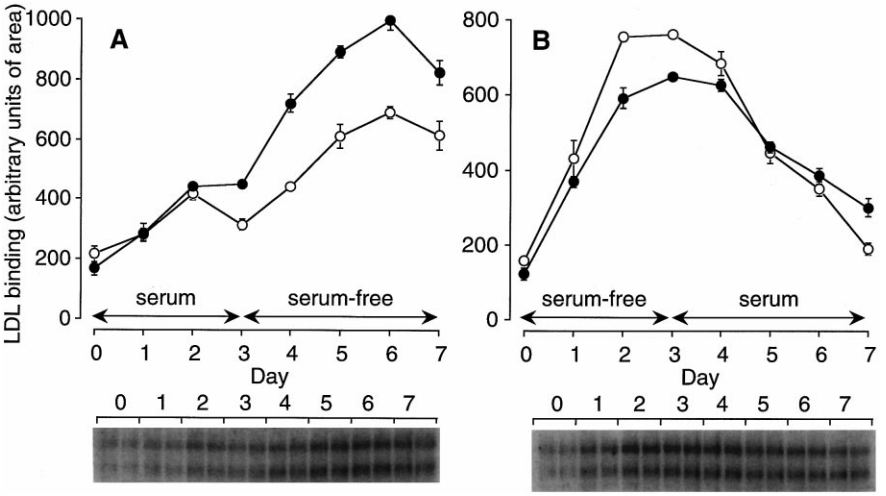

Fig. 2. Regulation of T-cadherin protein expression in SMC under serum-containing or serum-free culture conditions. Rat aortic SMC were seeded (at $\approx 0.3 \times 10^6$ cells/well) and cultured in the presence of 10% FCS for 24 h (day 0). Thereafter SMC were maintained for a further 3 days under either FCS-containing (panel A) or serum-free (panel B) conditions. On day 3, FCS-maintained cultures were changed to SF conditions (panel A), and SF-maintained cultures were switched back to FCS-containing conditions (panel B). Cell lysates were prepared on each day (0–7) and then electrophoresed in parallel. Data for levels p105 (●) and p130 (○) are given as mean ± S.D. of duplicate determinations from three separate experiments. Representative ECL detection images are presented.

they did not achieve a state of confluence within the first 3 days. Thus the cessation of increases in T-cadherin after 2 days under SF conditions (Fig. 2B) either reflects a maximal upmodulation of protein expression and/or is due to negative effects of autocrine growth factors that have accumulated in the medium. A striking difference between SF- and FCS-maintained cultures during the first 3 days is the relative increase in T-cadherin protein expression. This was much greater in SF-maintained cultures ($\approx$5-fold increase after 48 h, Fig. 2B) than FCS-maintained SMC ($\approx$2-fold increase after 48 h, Fig. 2A). Following a switch from FCS-containing to SF conditions (Fig. 2A), expression of T-cadherin proteins markedly increased, whereas the switch from SF to FCS-containing conditions (Fig. 2B) resulted in a downmodulation of protein expression.

The above data show a striking association between proliferation status of SMC and levels of T-cadherin expression, with upmodulation in quiescent or very slow growing cultures and downmodulation in cultures with high proliferation potential. Additional support of association between expression of T-cadherin and proliferation/density status in SMC might be derived from our consistent findings that the magnitude of alterations in p105 and p130 levels between SF and serum conditions is much greater in cultures of rat SMC (2.5–5-fold), which possess a rapid proliferation potential and achieve high saturation density, than in cultures of human SMC (1.3–1.5-fold, see Fig. 5), which possess a slow proliferation potential and achieve a low saturation density. Our findings with SMC are in accordance with a study [12] demonstrating that T-cadherin mRNA is present in normal epithelial cells but absent in tumor epithelial cells. Moreover, the same study demonstrated that reexpression of T-cadherin in mammary epithelial tumor cells by transfection markedly diminished their growth rate and that anti-T-cadherin antibodies blocked the growth inhibitory effect induced by T-cadherin reexpression [12].

3.3. *T-cadherin protein levels are not influenced by changes in cellular lipid/sterol metabolism*

Expression levels of all members of the LDL receptor (LDLR) family that function in cellular lipid transport/sterol metabolism are known to be variously upmodulated and downmodulated under conditions that influence cellular lipid metabolism ([18–23]; see also Fig. 5 for regulation of apo B,E

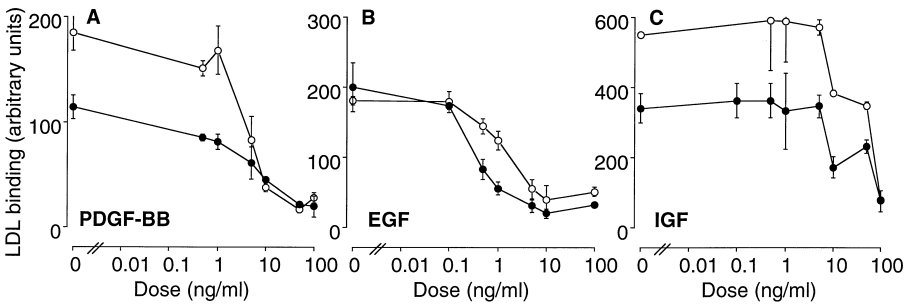

Fig. 3. Dose-dependent down-regulation of T-cadherin protein expression by growth factors. Rat aortic SMC were cultured to subconfluence, rinsed with SF medium and then further cultured in SF medium for 24 h either without (time 0) or with inclusion of the indicated concentrations of PDGF-BB (panel A), EGF (panel B), or IGF-1 (panel C). T-cadherin was measured in whole cell lysates as described in Section 2. Data for levels of p105 (●) and p130 (○) are given as mean ± S.D. of duplicate determinations from three separate experiments.

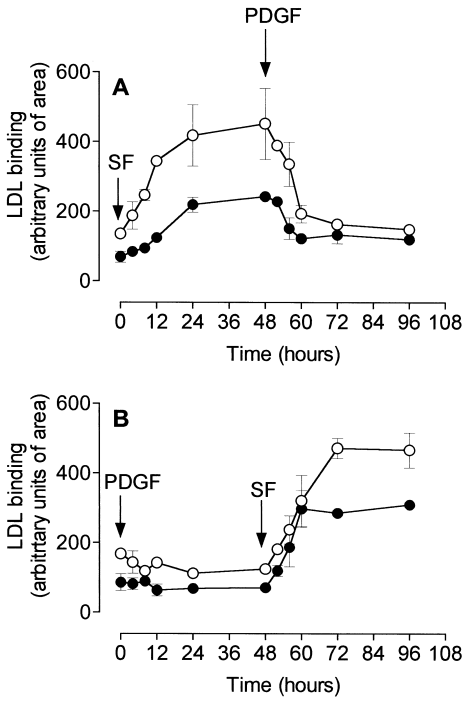

Fig. 4. Time-dependent alteration of T-cadherin protein expression in SMC cultured under serum-free conditions and in the presence of PDGF-BB. Rat aortic SMC were seeded ($\approx 0.5\times10^6$ cells/well) and cultured in the presence of 10% FCS for 24 h. After rinsing with SF medium (time 0) SMC were maintained either under SF conditions (panel A) or with inclusion of 10 ng/ml PDGF-BB (panel B). After 48 h, SF-maintained cultures (panel A) were changed to PDGF-BB-containing conditions (10 ng/ml PDGF-BB added every 12 h), and PDGF-BB maintained cultures (panel B) were changed to SF conditions. Cell lysates were prepared at the indicated time intervals. Data for levels of p105 (●) and p130 (○) are given as mean ± S.D. of quadruplicate determinations from two separate experiments.

receptor in human SMC). T-cadherin bears no sequence homology to the LDLR family proteins. Nevertheless, given that T-cadherin is an LDL-binding protein [3,4] we investigated whether serum-dependent downmodulation of T-cadherin might be due to effects on cellular lipid metabolism. Confluent SMC were treated for 24 h (under serum-containing and SF culture conditions) with LDL, HDL, VLDL (at 100 μg/ml), cholesterol (30 μg/ml), LPDS plus compactin (10% plus 3×10^{-5} M), dexamethasone (10^{-8}–10^{-5} M), progesterone (10^{-8}–10^{-5} M) or estradiol (10^{-8}–10^{-5} M). None of these conditions exerted conspicuous effects on either p105 or p130 levels in either rat or human SMC (complete data not shown; see Fig. 5 for clear distinctions between T-cadherin and apo B,E receptor). Serum-dependent downmodulation of T-cadherin thus involves serum-contained factors other than those which directly influence cellular lipid/sterol metabolism. Together with the cell surface location and outward orientation of T-cadherin [4,5], the lack of effect of lipid/sterol metabolism on T-cadherin protein levels can be interpreted to support that T-cadherin possesses some surface-directed function other than lipid endocytosis and lipid metabolism.

3.4. Reversible modulation of T-cadherin levels by SMC peptide growth factors

Specific serum-contained peptide growth factors, including epidermal growth factor (EGF, 1 ng/ml), platelet derived growth factor (BB homodimer, PDGF-BB, EC_{50} ≈5 ng/ml), and insulin-like growth factor-1 (IGF1, EC_{50} ≈10 ng/ml) elicited a dose-dependent downmodulation of T-cadherin (Fig. 4). The reversibility of this modulation was investigated by studying the kinetics of changes in p105 and p130 protein levels under SF and PDGF-BB (10 ng/ml)-containing culture conditions. For this, SMC were passaged and maintained normally (10% FCS) for 24 h (time 0) and then further cultured under either SF-to-PDGF (Fig. 4A) or PDGF-to-SF (Fig. 4B) regimens. In both regimens PDGF-BB was added repeatedly at 12-hourly intervals. Under the first regimen expression of p105 and p130 proteins could be detected within 4–8 h of serum deprivation and achieved maximal levels within 24 h; downmodulation of p105 and p130 expression in these cultures occurred promptly following addition of PDGF-BB, declining to minimal levels within 24 h and remaining at this level for at least the next 24 h (Fig. 4A). If PDGF-BB was added to cultures once only (i.e. at the 48-h time period) levels of p105 and p130 protein expression once again started to increase after the 72-h time period (data not shown). Under the second regimen in which SMC were first cultured in the presence of PDGF-BB, p105 and p130 levels remained suppressed until cultures were switched to SF conditions whereby expression of the proteins increased within 4–8 h, reached maximum within 24 h and was sustained over the next 24 h (Fig. 4B). As for the preceding regimen, repeated additions of PDGF-BB (i.e. at times 0, 12 and 24 h) were necessary to maintain a restricted expression of p105 and p130 proteins over a 48-h period (data not shown). The ability of specific SMC mitogens to downmodulate T-cadherin protein and the ready reversibility of downmodulation upon reversion to mitogen-free conditions provide further support for a close relationship between expression of T-cadherin and proliferation status of SMC.

3.5. Proliferation-dependent alterations in T-cadherin protein expression are specific

To ensure that changes in T-cadherin protein expression were specific and not artefacts of general changes in intracellular or membrane proteins we also investigated expression of

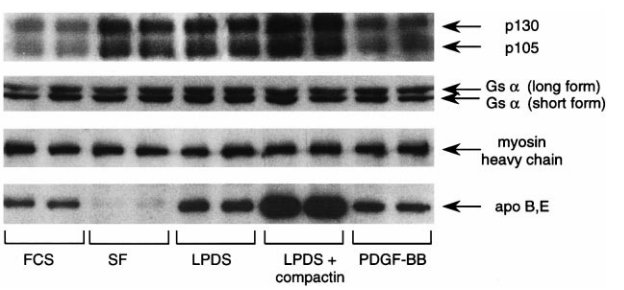

Fig. 5. Specificity of changes in levels of T-cadherin proteins: comparison with Gsα, myosin heavy chain and apo B,E-receptor. Human SMC were cultured to subconfluence, and serum-deprived for 48 h. Thereafter SMC were cultured for 24 h in the continued absence of serum (SF) or in the presence of FCS (10%), LPDS (10%), LPDS plus compactin (10% plus 3×10^{-5} M) or PDGF-BB (10 ng/ml). Four separate electrophoresis/electrotransfer runs were made, and blots were individually incubated for detection of either T-cadherin proteins (p105 and p130), Gsα, myosin heavy chain, or apo B,E receptor as described in Section 2. Representative ECL detection images are presented. Data from densitometric analysis is given in the text.

myosin heavy chain (control for cytoskeletal proteins) and Gsα (control for membrane proteins) in cell lysates from SMC grown under conditions in which p105 and p130 proteins are either minimally (plus serum, or PDGF-BB) or maximally (serum-free) expressed. In both human SMC (Fig. 5) and rat SMC (data not shown) levels of both myosin heavy chain and Gsα proteins remained constant under all experimental conditions (Fig. 5). Additionally, since the differences between minimal and maximal expression levels were consistently much smaller in human SMC (generally ≈1.3-fold, see Fig. 5 for example) than in rat SMC (≈2.5–5-fold, see Figs. 2–4), responsiveness of human SMC to different culture conditions was validated by concomitant examination of T-cadherin and apo B,E receptor (as positive control). In accordance with its established regulation [18,24], apo B,E receptor protein expression in human SMC was low under SF conditions, upregulated enormously (≈40-fold above SF) by a combination of LPDS plus compactin and to a lesser extent (≈5–10-fold above SF) by FCS, PDGF-BB and LPDS (see Fig. 5). In the same human SMC lysates T-cadherin protein levels varied as expected (i.e. elevated under serum-free conditions, and reduced in the presence of serum or growth factor). Apart from validating responsiveness of human SMC and the specificity of changes in T-cadherin protein levels, the data in Fig. 5 underline that apo B,E receptor and T-cadherin differ significantly with respect to their expression regulation, and therefore function.

3.6. Conclusion

We have presented data on in vitro regulation of expression of the lipoprotein-binding cell adhesion glycoprotein T-cadherin (p105 and p130) in SMC, which invoke some function(s) relevant to control of cell-cell contact (density-dependent expression) and growth (proliferation-status-dependent expression). The data are consistent with the notion that changes in the state of cell differentiation, proliferation and density often have an effect on the expression of adhesion molecules, and vice versa [6–9]. The mechanisms whereby levels of T-cadherin decrease or increase in response to a given culture condition need to be studied. Since T-cadherin levels were measured in whole cell lysates the reversibility of alterations in SMC cannot be attributed to regulation of surface expression via simple internalization and reappearance of the proteins. We can speculate that downmodulation reflects a real reduction of p105 and p130 proteins from the cell surface, via either negative transcriptional control or enzymatic cleavage, while upmodulation requires positive transcriptional control and de novo synthesis.

An additional important issue requiring investigation pertains to the physiological relevance of LDL binding to T-cadherin. We have previously demonstrated that LDL can promote SMC proliferation [26,27], and thus might speculate that the ability of LDL to serve as a specific, soluble heterophilic ligand for T-cadherin interferes with the homophilic T-cadherin interactions that are critical to normal tissue development and structure. Furthermore, information is emerging that in addition to their morphogenic function, cell adhesion receptors may also have important functions in signal transduction cascades (e.g. tyrosine kinases and protein kinase C) that impinge on the regulation of growth and differentiation [25]. It is not known whether T-cadherin function is linked to signalling pathways. However, LDL is capable of eliciting growth-associated intracellular signals in SMC [26–28] and our current objective is to investigate whether LDL-induced activation of signal transduction cascades is mediated via T-cadherin.

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