Human vascular smooth muscle cells (hVSMC) rendered quiescent by maintenance under serum-free culture conditions for 48 h exhibited several metabolic responses, normally associated with proliferation, following exposure to low density lipoprotein (LDL). LDL induced a time- and dose- (half-maximally effective concentration, ED_{50} 25.0 ± 8 nm) dependent activation of S_{6} kinase which could be negated following pretreatment of hVSMC with 12-O-tetradecanoylphorbol-13-acetate (TPA) for 48 h. In myo-[3H]inositol-prelabeled hVSMC, LDL caused a rapid (maximum within 1 min) decrease in phosphatidylinositol 4,5-bisphosphate (35% p < 0.001) and phosphatidylinositol 4-phosphate (20%, p < 0.01) with a return to prestimulated levels within 5–10 min. LDL induced a concomitant increase in [3H]inositol phosphates for which the order of generation was inositol-tris > bis > mono phosphate and which reached threshold levels of significance (p < 0.05) above control values within 1, 2, and 10 min, respectively. The effect of LDL on hVSMC phosphoinositide metabolism was dose-dependent (half-maximally effective concentration, ED_{50} 32.1 ± 5.0 nm). This concentration, like that for S_{6} kinase, approximates with the K_{D} (5–21 nm) for high affinity binding of 125I-LDL to specific receptors (1.5 × 10^{4} sites/cell) on hVSMC. LDL induced a rapid but transient translocation of protein kinase C from the cytosol to membranes as assessed by both immunoblotting and [3H]-4,6-phorbol-12,13-dibutyrate-binding procedures. Exposure of quiescent hVSMC to LDL elevated intracellular pH (Δ pH 0.30 ± 0.03, p < 0.001). Such alkalinization was prevented in the presence of Na^{+}/K^{+} exchange inhibitors such as amiloride, dimethylamiloride, and ethylisopropylamiloride. In an investigation of the nuclear action of LDL, a time-dependent induction of both c-myc and c-fos was observed. Such LDL-induced expression of these nuclear proto-oncogenes was not detectable in protein kinase C down-regulated hVSMC. Nevertheless, in spite of the cascade of “growth-promotional” responses elicited by LDL in quiescent hVSMC, this lipoprotein alone (under serum-free conditions) was neither mitogenic in nuclear labeling experiments, nor could it support growth of hVSMC in culture. We demonstrate that LDL might function in a complementary/synergistic fashion with other weakly mitogenic (to VSMC) growth factors and suggest that activation of protein kinase C (cis vs cis intrinsic tyrosine kinase characteristic of other growth factor receptors) may be crucial to the signal transduction pathway for LDL.

The stimulation of quiescent cells in culture to proliferate by serum and peptide growth factors has been shown to be dependent on a number of coordinated cellular events leading to DNA synthesis and cell division (1–4). One such event is the stimulation of S_{6} kinase activity which results in the reactivation of protein synthesis and is a prerequisite for cell division (5–7). We have previously studied S_{6} kinase activation (8–10) in vascular smooth muscle cells (VSMC) and have shown that some compounds, i.e. thrombospondin and angiotensin II (AII), which are not normally considered as growth factors for such cells, are capable of stimulating protein synthesis (8, 9). We have also confirmed the findings of Pouysségur et al. (11), namely that S_{6} kinase activation and elevation of intracellular pH are intimately associated (9). However, although in our hands neither AII nor thrombospondin were mitogenic to VSMC under serum-free conditions, both compounds were capable of eliciting a number of important proliferative intracellular responses suggesting that in combination with other factors they may be growth promoters in vivo. In particular, AII has been shown to stimulate expression of the c-fos proto-oncogenes in VSMC in an analogous manner to fetal cell serum (12), and we and others have confirmed that thrombospondin in combination with EGF markedly stimulates DNA synthesis in VSMC (13, 14).

There has been a prolonged interest in the role of low density lipoproteins (LDL) in VSMC proliferative behavior (15–18). Some evidence suggests that LDL is mitogenic to VSMC (16) while other studies have shown that the same compound only supplies essential lipids for growth (18). These discrepancies mostly relate to the culture conditions/treatments under which the effect of LDL was tested. More recently, Chen et al. (19) observed stimulation of growth of VSMC only by intact LDL particles and not the separate lipid and/or apoprotein components, which suggests to us that LDL may indeed play an important promotional role to VSMC proliferation.

We therefore studied the effects of LDL on quiescent human VSMC under serum-free conditions. Although LDL

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The abbreviations used are: VSMC, vascular smooth muscle cells; AII, angiotensin II; LDL, low density lipoprotein; PDBu, 4P-phorbol-12,13-dibutyrate; TPA, 12-O-tetradecanoylphorbol-13-acetate; FCS, fetal calf serum; DMO, 5,5-dimethyl-oxazolidine-2,4-dione; EGF, epidermal growth factor; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitriolo)tetracetic acid; MOPS, 4-morpholino-propanesulfonic acid; Ptd-Ins, phosphatidylinositol; Ptd-InsP_2, phosphatidylinositol bisphosphate; Ptd-InsP_3, phosphatidylinositol trisphosphate; DMA, dimethylamiloride; EIPA, ethylisopropylamiloride.
alone neither increased proliferation nor stimulated DNA synthesis in our VSMC preparations under test conditions, we did observe that low levels of LDL induced a number of intracellular responses normally associated with growth factor stimulation of cells. Furthermore, under serum-free conditions, pretreatment of human VSMC with 12-O-tetradecanoylphorbol-13-acetate (TPA) completely negated any subsequent activation by LDL suggesting that the effects of LDL were mediated via protein kinase C.

**EXPERIMENTAL PROCEDURES**

**Materials**

All materials and media for tissue culture were obtained from Gibco AG, Basel, Switzerland, except for fetal calf serum (FCS) which was purchased from Palka AG, Basel, Switzerland. **[^1]** Iodine carrier free was from Du Pont-New England Nuclear, Zürich, Switzerland. All other radioisotopes used in this study were from Amersham, Zürich, Switzerland, and they included molybdenum-125 (16 Ci/mmol), [gamma-32P]ATP (3000 Ci/mmol), [methyl-3H]thymidine (92 Ci/mmol), [carbon-14]glucose (50 mCi/mmol), 5,5-dimethyl-[2-14C]oxazolidine 2,4-dione (DMO) and [4,4,5,5-tetramethyl-3H]thymidine (TIDBu).

The same company supplied monoclonal antibodies for protein kinase C (clone MC5), LDL receptor (IgGC) and factor VIII antigen. Cold protein A-Sepharose 4B (anti-CD) were obtained from Dakopatts, Denmark. All other chemicals and reagents were purchased from E. Merck, Darmstadt, Federal Republic of Germany, Sigma, or in the case of unlabeled DMO, Aldrich. Reagents for RNA procedures were purchased from Bethesda Research Laboratories and were of highest grade obtainable.

**Methods**

**Isolation and Culture of Vascular Smooth Muscle Cells**—The isolation and culture of human vascular smooth muscle cells (hVSMC) was performed essentially as described previously for rat VSMC (20); the tissue of origin was obtained from patients undergoing abdominal surgery and consisted of microarterioles associated with omental fat. Tissue was extensively cleaned to remove all connective tissue by micro dissection and then finely minced prior to enzymatic dissociation (20). Cell isolates were plated onto extracellular matrix-coated plastic ware (21, 22) and cultured as described (20). When primary cultures reached confluence (7-9 days, depending on seeding density) cells were redistributed by trypsinization and fresh cultures initiated. Cultures reached confluence (7-9 days, depending on seeding density) cells were harvested and disrupted by sonication. The homogenate fraction was taken to represent total (100%). The cytosol fraction was prepared by centrifugation of homogenates at 100,000 X g for 30 min at 4 °C. Supernatants (cytosol) were withdrawn before suspension and sonication of pellets (membrane) in 500 μl of PKB. Samples were stored at −70 °C until use. Phorbol 12,13-didecanoate (PDBu) ester binding was performed essentially as described previously (31) in the presence and absence of 10μM PDBu. Sample aliquots were incubated (overnight at 4 °C with vigorous shaking) in a reaction mixture (250 μl) containing 20 mM Tris-HCl, pH 7.4, 10 mM Mg(NO3)2, 1 mM CaCl2, 400 μg/ml phosphatidylserine, 4 mg/ml bovine serum albumin, and 50 mM [3H]PDBu, and thereafter [3H]PDBu binding determined by filtration through Whatman GF/C glass fiber filters. Intracellular pH was determined following exposure of quiescent hVSMC to 20% (v/v) fetal calf serum or 0.1 μM TPA.

**LDL-induced alkalinization in human VSMC**

Intracellular pH was determined following exposure of quiescent hVSMC to 20% (v/v) fetal calf serum or 0.1 μM TPA. Values are mean ± SEM. (From four separate experiments, triplicate determinations). pH represents the change in intracellular pH relative to that in serum-free conditions where the asterisk denotes a significant (p < 0.001) increase in pH.

| Conditions | Intracellular pH | ΔpH |
|------------|------------------|-----|
| Serum-free | 6.67 ± 0.07      |     |
| 10% FCS    | 6.94 ± 0.02      | 0.36 ± 0.02* |
| 0.1 μM TPA | 7.30 ± 0.09      | 0.61 ± 0.04* |
| 0.01 μM 4α-phorbol | 6.68 ± 0.09 | 0.01 ± 0.00  |
| 0.005 μM LDL | 6.97 ± 0.15 | 0.20 ± 0.03  |
| 51 μg/ml LDL + 0.5 mM amiloride | 5.91 ± 0.17 | -0.64 ± 0.17 |
| 51 μg/ml LDL + 1 μM EIPA | 6.16 ± 0.06 | -0.52 ± 0.18 |
| 51 μg/ml LDL + 100 μM DMA | 6.46 ± 0.03 | -0.09 ± 0.03 |
using monoclonal antibody to protein kinase C (clone MC5, Amersham Corp.) and 0.2 μCi/ml[^125I] labeled (Amersham Corp.) sheep anti-mouse IgG as the second antibody. Immunodetectable protein kinase C was quantitated after location by autoradiography, excision of material from membranes, and counting in a multwell gamma-counter. All values were normalized with respect to protein (30-40 μg) loaded on the gels.

Measurement of Proto-oncogene Induction—Quiescent hVSMC, grown in 150 mm dishes, were exposed to either FCS (20%), LDL (100 nM), or TPA (100 nM) for times indicated in the relevant results section. Stimulated cells were harvested by trypsinization. After a single wash in phosphate-buffered saline cells were lysed in GT-buffer (34) and RNA was collected by centrifugation of the lysate on a 5.7 M CsCl gradient at 35,000 rpm in an SW50 rotor for 12-16 h (34).

For Northern blotting 20 μg of total RNA was electrophoresed through an 1.2% Agarose gel containing 2 M formaldehyde. Gels were run at 50 V for 6-8 h in MOPS buffer and blotted to Hybond nylon membranes (Amersham Corp.) with 20 X SSC (sodium chloride, sodium citrate) as blotting buffer. Subsequently, membranes were removed, washed briefly in 2 X SSC, dried for 10 min at 80 °C, and then RNA was fixed to membranes by UV irradiation for 3 min at 302 nm. Blots were hybridized to random primed c-myc (35) and c-fos (36) probes according to the method of Gilbert and Church (37). Exposure of blots to Kodak X-Ormat films was done overnight at -70 °C with the use of one intensifying screen.

Assay for DNA Synthesis—Assays of DNA synthesis were performed on quiescent hVSMC (serum deprivation for 48 h) plated into costar 12-well multiwell dishes as described previously (38) using [3H] thymidine.

Protein Determination—Protein concentration was determined using the method of Bradford (29), with bovine serum albumin as standard.

RESULTS

Specific Binding of LDL to Intact Cells—Quiescent human vascular smooth muscle cells (hVSMC) exhibited specific and saturable binding for[^125I] labeled LDL (Fig. 1). Scatchard transformation of the data (inset, Fig. 1) indicated that hVSMC possessed high affinity (K_D ranged from 0.5 to 2.1 x 10^-8 M) and low affinity (K_D > 10^-10 M) sites. The number of high affinity LDL receptors found on hVSMC in three separate experiments was ~1.5 x 10^6 sites/cell.

Preincubation of cultures with C1 monoclonal antibodies (I_GC) against specific LDL surface receptor (26) reduced subsequent binding of[^125I] labeled LDL by ~60%. I_GC was used at between 10 and 50 nM in three binding experimental protocols.

Activation of S_6 Kinase by LDL—LDL induced a time-dependent activation of S_6 kinase in quiescent hVSMC (Fig. 2). The time course for this activation was similar to that previously observed following exposure of quiescent rat aortic VSMC (9), or hVSMC to TPA (not shown). These kinetics are characterized by a relatively slow maximum after (30 min) and more sustained activation of S_6 kinase as opposed to the rapid maximum (after 10 min) and transient activation profiles observed in response to growth factors (e.g. EGF (7, 8)) and vasoconstrictors (e.g. AII (9)). LDL-induced activation of S_6 kinase was dose-dependent and the half-maximally effective concentration of LDL was calculated to be 25.0 ± 8 nM (Fig. 3A). This is comparable to the K_D value obtained for LDL binding to high affinity sites. The ability of TPA to activate S_6 kinase in quiescent hVSMC was negated when...
To 3′-phosphoinositide  and diacylglycerol (40, 41). We  therefore  that LDL-induced  stimulation of s6 kinase  might be mediated  by protein  kinase C since  this enzyme specifically is consid-
ered  to be the cellular  phorboid receptor  (39, 40).

Since protein kinase C is dependent on diacylglycerol (and Ca^{2+})  for activation, its participation in the signal transduc-
tion pathway for LDL would imply hydrolysis of phosphatidy-
linositol-bisphosphate  and consequent generation of inosi-
tol trisphosphate and diacylglycerol (40, 41). We therefore  investigated the ability of LDL to elicit catabolism of phos-
phoinositides in myo-[3H]inositol prelabeled VSMC. Fig. 4  shows the changes in three [3H]inositol phospholipids (Ptd-Ins,  Ptd-Insp,  Ptd-InspP) of hVSMC at various times after  addition of LDL. There was a rapid, transient decrease in [3H] content of Ptd-Insp (by ~20%, p < 0.01 after 1 min) and  Ptd-InspP (by ~35%, p < 0.002 after 1 min) with a return to prestimulated levels within 5–10 min. There was little change  in Ptd-Ins with 3H content decreasing to significance (~10%,  p < 0.05) only after 15 min of exposure to LDL.

Fig. 5 depicts LDL-induced generation of [3H]inositol phos-
phates for which the order of initial rates of generation of  Ins-P3 > Ins-P > inositol phosphate. Increases in these inositol  phosphates above control values reached threshold levels of  significance (p at least <0.05) within 1, 2, and 10 min, respectively. Maximum increases in Ins-P3 and Ins-P2 occurred  between 5–10 min after which time [3H] content de-
clined, whereas the increase of [3H] content in Ins-P was  sustained (in the presence of 15 mM LiCl). The kinetics of  LDL-induced phosphoinositide turnover, with respect to both  phospholipid degradation and inositol phosphates, are in  agreement with that established for other phosphatidylinosi-
tol-mobilizing hormones (27). The half-maximally effect-
ive concentration of LDL for generation of [3H]-inositol tri-
phosphate was calculated to be 32.1 ± 5.0 nM. This concen-
tration, like that for Ss kinase activation, corresponds to the  Kd of hVSMC for high affinity LDL binding.

Translocation of Protein Kinase C—TPA and a variety of  hormones and growth factors can induce the translocation of  protein kinase C from the cytosol to membranes, and this is  believed to reflect the intracellular activation of the enzyme  (31, 42). Using monoclonal antibodies to protein kinase C, we
Phosphoesters were separated by anion-exchange chromatography ("Methods"). Data are given as means ± S.D. from duplicate determinations for each time point in two separate experiments. Immunodetectable protein kinase C from the cytosol to the membrane in hVSMC. At quiescence the distribution (% of total in homogenate) of [3H]PDBu binding between membrane and cytosol fractions was 38 ± 5% and 70 ± 6%, respectively (Fig. 7). Following exposure to LDL the % distribution of [3H]PDBu in membranes increased with a concomitant and inversely proportional (% decrease) change in cytosolic [3H]PDBu binding (Fig. 7). Such translocation induced by LDL was maximal within 2 min whereafter a retranslocation of [3H]PDBu binding occurred which remained stable with prolonged exposure (up to 30 min) to LDL.

Amiloride-sensitive Alkalization—Both protein kinase C and S6 kinase activation are intimately involved in the onset of proliferation of quiescent cells, and their stimulation is associated with a rapid intracellular alkalization brought about via activation of the Na+/H+ antiporter (4, 11, 39, 42, 44). Exposure of quiescent hVSMC to LDL elevated intracellular pH with the ∆pH, being similar to that measured in the presence of 10% FCS (Table I). TPA but not the 4α-phorbol isomer also increased pH. The effects of LDL on hVSMC pH were prevented in the presence of Na+/H+ exchange inhibitors such as amiloride and amiloride analogues, DMA or EIPA (Table I). Significant (p at least <0.05) acidification was in fact observed in the presence of these compounds.

Specificity of LDL for Stimulation of Signal Transduction—To ensure that the induction of the metabolic events in hVSMC described above were specific to LDL, we performed a number of experiments (n = 3) using antibodies against either apolipoprotein B (antiapo B) or LDL cell surface receptors (IgGc, 7). Pretreatment of cells with IgGc (30 nM) for 2 h at 4 °C, prior to their exposure to LDL, reduced (by -60%) their response to the agonist in terms of stimulation of phosphoinositide turnover, S6 kinase activation, and elevation of intracellular pH. Further inhibition (~10%) was observed if additional fresh IgGc antibody was included with LDL during

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stimulation procedures at 37 °C (see Methods). Smooth muscle cells have been shown to rapidly internalize bound C₇ antibodies at 37 °C (26). The level of inhibition by C₇ of the signal transduction processes studied was comparable to that observed for ¹²⁵I-labeled LDL binding to antibody-treated cells (results above - LDL binding). When LDL (10-50 µg of protein) was preincubated (4 °C for 2 h) with antiapo B (12 µg of protein) prior to addition to quiescent hVSMC in stimulation assays (phosphoinositide turnover and S₆ kinase activation) we observed a decreased (range 60-28% of control values) level of induction of these pathways as compared with control cultures treated with antiapo B only.

The levels of stimulation of phosphoinositide metabolism and S₆ kinase activation by AII in cultures of quiescent hVSMC were not reduced by either antibody preparation.

**Nuclear Proto-oncogene Expression, Mitogenesis, and Growth**—The data thus far presented demonstrate that in hVSMC, LDL elicits several biochemical responses common to the action of a variety of recognized growth factors (41, 44). Increasing attention is being focused on the possible role of hormones in regulating cell proliferation, and of particular interest to VSMC in this regard is the vasoconstrictor angiotensin II. In addition to the ability of AII to simulate phosphoinositide catabolism and elevate intracellular pH (27, 45), this hormone has recently been demonstrated to activate S₆ kinase (9) and to induce expression of the nuclear protooncogenes c-myc and c-fos (12, 46). We have thus investigated the nuclear actions of LDL. Exposure of hVSMC to LDL resulted in a time-dependent stimulation of both c-myc and c-fos expression, although the levels of expression were somewhat smaller than those induced by 10% (v/v) FCS (Fig. 8). The time courses for induction were identical for TPA and LDL. After pretreatment of hVSMC with TPA for 48 h, a time-dependent induction of c-myc expression by LDL was not discernable. However, some induction of c-fos remained under these conditions. The induction of the two protooncogenes by re-exposure of pretreated cells to TPA was also not apparent (Fig. 8B). Pretreatment of quiescent cultures with TPA did not, however, adversely affect the inductive capacity of 10% (v/v) FCS (Fig. 8B).

To investigate the growth promotional properties of LDL, hVSMC were subjected to long-term exposure to LDL under serum-free conditions. No increase in cell number was evident and the stimulation of [³H]thymidine incorporation into DNA was very small by LDL alone (Fig. 9). However, in combina-

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**Fig. 8.** Kinetics of induction of c-fos and c-myc transcription in hVSMC by FCS, LDL, and TPA. Two separate isolates of quiescent hVSMC (H₆ and H₁₀) were exposed to either FCS (10%), LDL (100 nm), or TPA (100 nM) for the times shown in the figure and the induction of c-fos (2.2 kilobases) and c-myc (2.7 kilobases) transcription assessed by Northern analysis (see "Methods"). Induction experiments were performed either before (A) or after (B) cells had been pretreated for 48 h with TPA (100 nM). Experiments were repeated with each isolate using different preparations of LDL.

**Fig. 9.** Combined comitogenic effects of LDL and EGF on hVSMC. Cells suspended in normal culture medium (Methods) were plated into Costar 12-well multidwell plates at either (a) 2 x 10⁵ cells/well or (b) 10⁶ cells/well, and after 18 h medium was replaced by serum-free medium or the same containing either LDL (100 nM), EGF (10), or a combination of the same (1). Agonists were used at 10⁻¹ and 10⁻⁸ M, respectively, both alone and in combination. Cell numbers (a) were determined at the times shown as described previously (20), and cultures were given daily medium changes. The data represents the means ± S.D. of values obtained on experiments (n = 4) using the two cell isolates described. The stimulation of [³H]thymidine incorporation into DNA (b) was performed as described (38) on quadruplicate wells for each treatment: cells were maintained for 48 h (with one change) on serum-free medium prior to exposure to agonists in the same medium containing [³H]thymidine ("Methods"). The data represents the means ± S.D. (after subtraction of serum-free values) for the level of incorporation of isotope into DNA and are expressed as dpm/10⁶ cells. The level of incorporation exhibited under block LDL/EGF was that obtained experimentally and was significantly (p < 0.001) higher than the theoretical value. Experiments were performed a number of times (n = 3) with each cell isolate.
tion with EGF, itself a weak mitogen for VSMC (13), we observed both sustained, albeit slow, growth of hVSMC and a significant stimulation of DNA synthesis (Fig. 9). Indeed combinations of LDL and EGF resulted in a synergistic stimulation of DNA synthesis (compare LDL/EGF, with A; Fig. 9b).

**DISCUSSION**

This study demonstrates that LDL is capable of activating, in human vascular smooth muscle cells, a number of cellular events which are intimately linked to the onset of proliferation in quiescent cells (41, 44). These processes include phosphoinositide turnover, activation of \( S_6 \) kinase, alkalization, translocation of protein kinase C, and induction of nuclear proto-oncogene expression.

Furthermore using specific antibodies to either LDL (antiapo B) or its cell surface receptor, \( _{1} \)GC\(_{7} \) (26) we have been able to demonstrate that the observed responses are specific to LDL and are receptor mediated. To obtain complete inhibition of the LDL stimulations of hVSMC using the \( C_7 \) receptor antibody was difficult since the latter is rapidly internalized at \( 37^\circ C \) during incubation of cells with LDL for stimulatory purposes. Nevertheless, the 60% reduction in binding of \( ^{113} \)T-labeled LDL to \( _{1} \)GC\(_{7} \)-treated cells was paralleled by a similar reduction in their stimulation (\( S_6 \) kinase, phosphoinositide turnover) by LDL. Prior treatment of LDL preparations with monoclonal antibody to platelet-derived growth factor at levels that inactivated responses due to this growth factor (8) did not reduce the stimulatory effects of LDL on hVSMC in our studies. We have also shown that platelet-activating factor used at levels that have been shown to be present in LDL preparations of others (47) has invoked the involvement of protein kinase C in cytoplasmic Mg\(^{2+} \) regulation. Since intracellular alkalization is a prerequisite for agonist-induced activation of \( S_6 \) kinase (4, 9, 11), it is not surprising that LDL also elevated intracellular Mg\(^{2+} \) in hVSMC. Our experiments with amiloride and the amiloride analogues, DNA and EIPA, indicated that the increase in pH is mediated via stimulation of the amiloride-sensitive Mg\(^{2+} \)/H\(^{+} \) antiporter as has been found for TPA and growth factors/hormones (9, 11, 41, 43, 45, 52). Such a metabolic response is believed to play a significant role in induction of cell proliferation and division (39, 41), phosphoinositide signal transduction (53), and regulation of muscle contractility and tone (43).

Our results further substantiate the findings of others in relation to nuclear proto-oncogene activation in cultured VSMC by mitogenic agents (36, 54) and also underline the potential growth-promotional properties of LDL. The observation that the time course for c-myc induction by LDL mimicked that of TPA (Fig. 8) and differed substantially from that of FCS and other mitogenic compounds make it unlikely that the effects of LDL reported here are due to contamination of our preparations with such agents. Although we observed a low constitutive expression of c-myc in our cultures maintained on serum-free medium, we are unable to say if such transcripts are functionally active. They may represent the pool of more stable, non-polyadenylated c-myc mRNA molecules recently reported by others (55). The detailed mechanism of c-fos induction is still not fully elucidated, although in the case of FCS it appears that specific serum response elements in the promoter region of the gene are important for this process (56, 57). The same is not true for c-fos activation by TPA, working as it does through protein kinase C (58). The fact that we still observed some residual activation of c-fos by LDL following prolonged pretreatment of hVSMC with TPA suggests that either an alternative pathway exists for LDL to exert this effect, or activation of c-fos occurs as a consequence of stimulation of other intracellular events by LDL as already discussed above.

In spite of the ability of LDL to promote such a wide spectrum of characteristic mitogenic cellular responses, we found that LDL alone is neither mitogenic to hVSMC nor would it support these cells in culture over a long period (5-7 days) under serum-free conditions. Clearly, therefore, it cannot be defined as a true mitogen for VSMC, but that this lipoprotein does elicit a cascade of growth promotional responses would suggest that its role is more important than mere supply of lipids essential for growth (18).

We propose and our data (Fig. 9) support the idea that LDL may play a complimentary role with other growth factors
along the lines already shown for thrombospondin with epidermal growth factor (13, 14). Thrombospondin alone, like LDL, will not promote growth or appreciably stimulate DNA synthesis but in the combined presence of epidermal growth factor, a weak mitogen for VSMC, a marked synergistic proliferative response is observed (13, 14). Our findings with combinations of LDL and EGF (Fig. 9 and Ref. 38) lend further credence to the importance of growth factor/growth promoter complementation. Such interactions have also been proposed for oncogenes (41). We are currently investigating possible mitogenic interactions between LDL and other well-known mild VSMC mitogens.

A fundamental question remaining concerns the importance of the stimulatory properties of LDL reported herein in relation to the inappropriate and deregulated growth of VSMC in vascular pathology. Evidence is accumulating for the trapping and localized concentration of LDL within the extracellular milieu of the blood vessel wall (59–61). The relevance of the ability of LDL to activate proliferative responses may reside in this area because of the influence that matrix macromolecules exert on several cellular processes including proliferation (62). Furthermore, the once simplistic view that growth factors were specific for a single cell type and could function independently is no longer tenable (15). The trapping and localized concentration of LDL within the matrix macromolecules exert on several cellular processes including proliferation (62). Furthermore, the once simplistic view that growth factors were specific for a single cell type and could function independently is no longer tenable (15).

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REFERENCES

1. Holey, R. M. (1975) Nature 258, 487–490
2. Jimenez de Asua, L., Clinigian, D., and Rudland, P. S. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2724–2728
3. Thomas, G., Martin-Perez, J., Settman, M., and Otto, A. M. (1982) Cell 30, 235–242
4. Chambard, J.-C., and Pouyssegur, J. (1986) Exp. Cell Res. 164, 282–294
5. Brooks, R. F. (1977) Cell 12, 311–317
6. Kiselinsky. R. Treloar, M. A., and Weiler, L. (1984) J. Biol. Chem. 259, 1351–1356
7. Novak, M., and Thomas, G. (1986) J. Biol. Chem. 261, 10314–10319
8. Scott-Burden, T., Resink, T. J., Burd, U., Burgin, M., and Buhler, F. R. (1986) Biochim. Biophys. Acta 866, 299–306
9. Scott-Burden, T., Resink, T. J., Burd, U., Burgin, M., and Buhler, F. R. (1986) Biochim. Biophys. Acta 851, 389–398
10. Scott-Burden, T., Resink, T. J., Burd, U., Burgin, M., and Buhler, F. R. (1989) Hypertension 13, 255–304
11. Pouyssegur, J., Chambard, J.-C., Franchi, A., Peris, S., and Van Obberghen-Schilling, E. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3935–3939
12. Kawahara, Y., Nakano, M., Tsuda, T., Fukuzaki, H., Fukumoto, Y., and Takai, Y. (1986) Biochem. Biophys. Res. Commun. 130, 52–59
13. Majadik, R., Cool, S. C., and Borestein, F. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9050–9054
14. Scott-Burden, T., Resink, T. J., and Buhler, F. R. (1988) J. Cardiovasc. Pharmacol. 12, 124–127
15. Ross, R., and Glomset, J. A. (1973) Science 180, 1332–1338
16. Fries, G. M., Kirchhausen, T., Fischer-Dzega, K., Wissel, R. W., and Scam, A. M. (1982) Atherosclerosis 41, 171–183
17. Augustyn, J. M., Fritz, K. E., Deacon, A. S., and Jermolavic, J. (1977) Atherosclerosis 27, 179–188
18. Libby, P., Miao, P., Orgadis, J. M., and Schara, E. J. (1986) J. Cell Physiol. 124, 1–8
19. Chen, J.-K., Hoshi, H., McCue, D. B., and McKeehan, W. L. (1986) J. Cell. Biol. 109, 207–214
20. Resnik, T. J., Scott-Burden, T. B., and Buhler, F. R. (1987) Hypertension 5 (Suppl. 5), 207–214
21. Jones, P. A., Scott-Burden, T., and Gevers, W. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 553–557
22. Scott-Burden, T., Bogenmann, E., and Jones, P. A. (1980) Exp. Cell Res. 156, 227–230
23. Atherosclerosis, J. H., and Buhler, F. R. (1986) Trends in Pharmacol. Sci. 9, 94–99
24. Moses, H. L., Coffey, R. J., Leef, E. B., Lyons, R. M., and Messing, G. A. (1977) J. Cell Biol. 75, 1–17
25. Sporn, M. B., and Roberts, B. A. (1980) Nature 284, 307–317
26. Brown, M. S., L豚h, A. W., and Buhler, F. R. (1988) Atherosclerosis 75, 263–269
27. Scott-Burden, T. and Buhler, F. R. (1986) Trends in Pharmacol. Sci. 7, 75–79
28. Atherosclerosis 30, 117–128
29. Pouyssegur, J., and Buhler, F. R. (1986) Trends in Pharmacol. Sci. 7, 75–79
30. Scott-Burden, T., Scott-Burden, T. A., and Buhler, F. R. (1986) Trends in Pharmacol. Sci. 7, 75–79