LDL Receptors in Keratinocytes

Maria Ponec, Marinus F.W. te Pas, Louis Havekes, Johannes Boonstra, A. Mieke Mommaas, and Bert Jan Vermeer
Department of Dermatology, University Hospital Leiden, Gaubius Laboratory (LH), IVYO-TNO, Leiden; and Department of Molecular Cell Biology (JB), University of Utrecht, The Netherlands

The presence of sufficient amounts of cholesterol in the epidermis is necessary for proper functioning of plasma membranes in the viable epidermal cell layers and also for the barrier quality of lipid intercellular bilayers of the stratum corneum. Cholesterol can be generated by local epidermal synthesis, or imported from the circulation as low-density lipoprotein (LDL), which is internalized by the cells by receptor-mediated endocytosis. Because of the complex structure of the skin, a model consisting of cultured human keratinocytes has been used to study in detail the regulation of epidermal sterologenesis in relation to keratinocyte differentiation. Experimental modulation of the differentiation of normal human keratinocytes has been achieved by varying extracellular calcium concentration or by comparison of a number of human squamous carcinoma cell lines and normal keratinocytes. These studies have clearly demonstrated a reciprocal correlation between the ability of cells to differentiate and LDL receptor activity. Regulation of LDL receptor expression has been found to occur at the DNA, mRNA, and protein levels, depending on the cell line studied. In normal but not malignant keratinocytes, the induction of keratinocyte differentiation was associated not only with a decrease of functional LDL receptors but also with changes in their cellular distribution. This conclusion is drawn from the observations that only in normal human keratinocytes, cultured at physiologic calcium concentrations, high levels of intracellular, cytoskeleton-associated receptors were found. Differentiation-related modulations of the LDL-receptor expression and of the cellular LDL-receptor distribution found in cultured keratinocytes were in agreement with observations made in the epidermis in situ. J Invest Dermatol 98:50S–56S, 1992

Mammalian cells can derive their cholesterol, a key component of the plasma membrane, either by the uptake from lipoproteins present in blood or by de novo synthesis within the cells [1,2]. In the last decade abundant information has accumulated concerning the uptake of lipoproteins and the role of membrane receptors in this process (reviewed in [1,2]). The specific serum lipoprotein particle, which normally supplies cholesterol to cells, is low-density lipoprotein (LDL). Following LDL binding to high-affinity receptors located on the plasma membrane, the receptor-ligand complexes cluster into coated pits and are subsequently internalized. Within endosomes, the LDL particle and its receptor dissociate, and a portion of the receptor molecules is recycled to the cell membrane. Degradation of LDL occurs after the fusion of endosomes with lysosomes. Released cholesterol influences three cellular processes: i) suppression of de novo sterologenesis through inhibition of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase; ii) increase in the storage of cholesterol as cholesteryl esters by activation of the enzyme acyl-CoA: cholesterol acyltransferase (ACAT); and iii) suppression of LDL-receptor synthesis [2].

LDL Receptor The LDL receptor is a glycoprotein of 858 amino acids consisting of five domains (Fig 1) [3,4]. Three of these domains are extracellular (comprising in total 750 amino acids), the fourth domain is transmembrane, and the fifth domain is cytoplasmic. The first extracellular domain, which most probably contains the ligand binding site for LDL, encompasses the N-terminal 292 amino acid residues of the protein and comprises seven copies of a cysteine-rich, negatively charged peptide of 40 amino acids. This domain exhibits a strong homology with the amino acid sequence of the human terminal complement component C9 [3]. The second domain comprises approximately 400 amino acids and shows homology to the precursor of epidermal growth factor (EGF), including three cysteine-rich repeats known as growth factor-like repeats. The third extracellular domain of 58 amino acids appears to be rich in glycosylated hydroxylated amino acids (serine and threonine) and is most probably important for the maintenance of receptor protein structure. The membrane-spanning domain contains 22 hydrophobic amino acids connecting the extracellular domains with the cytoplasmic domain. Finally, the cytoplasmic domain of the LDL receptor is the COOH-terminal part of 50 amino acid residues. The newly translated protein also contains a cleavage signal sequence that directs the newly synthesized protein to the cell surface [2].

For proper functioning it is necessary that the LDL receptor possesses i) specific extracellular high-affinity binding sites for its ligands Apo B and Apo E; ii) recognition signal for movement of the receptor into a clathrin-coated pit to enable the endocytosis of the receptor and the ligand; and iii) structural features that regulate the recycling of the receptor to the cell surface and ensures delivery of the LDL to lysosomes for degradation and alternatively for the degradation of the LDL receptor itself. Extensive studies [5,6] in the past years have revealed that the binding of LDL to the receptor requires the presence of 6 of the 7 cysteine-rich repeats in the first domain (Fig 1), whereas for the

Reprint requests to: Dr. Maria Ponec, Department of Dermatology, University Hospital Leiden, P.O. Box 9600, 2300 RC Leiden, The Netherlands.

Abbreviations:
ACAT: acyl-CoA: cholesterol acyltransferase
β-VLDL: very low density lipoprotein
EGF: epidermal growth factor
FCS: fetal calf serum
HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A
LDL: low-density lipoprotein
LPDS: lipoprotein-deficient serum
SCC: squamous carcinoma cells

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human epidermal keratinocytes offer an attractive alternative to the human skin in vivo, as the degree of keratinocyte differentiation can be experimentally manipulated by 1) modulation of extracellular calcium concentration and of cell density; 2) the use of transformed keratinocytes with defects in their terminal differentiation program; and 3) culturing of keratinocytes at the air-liquid interface. All studies performed till now clearly show that both LDL-receptor expression and the extent of LDL-regulated sterologenesis are inversely related to the capacity of keratinocytes to differentiate.

MATERIALS AND METHODS

Cell Culture Juvenile foreskin keratinocytes derived from donors (aged 1–2 years) and SCC lines (SCC-4, SCC-15, SCC-12F2) [11,12] were cultured together with irradiated mouse 3T3 fibroblast feeder cells in DMEM and Ham's F12 (3:1) media supplemented with 5% fetal calf serum (FCS), 0.4 μg/ml hydrocortisone. Normal keratinocytes were supplemented with 10–6 M isoproterenol and 10 ng/ml epidermal growth factor (EGF) [13]. For low-calcium conditions, calcium-free DMEM was mixed with standard Ham's F12 (3:1) medium and supplemented with 5% cholextreated fetal calf serum [14] and other additives as listed above. The final calcium concentration was 0.06 mM as determined by flame photometry.

Cell Association and Degradation of 125I-Labeled LDL Cells were preincubated for 24 h with medium containing lipoprotein-deficient newborn calf serum (LDPS) to obtain expression of LDL receptors. 125I-labeled LDL was then added (10 μg/ml), and the cells were reincubated for 3 h at 37°C. For assessment of nonspecific binding, 300 μl of unlabeled LDL was added to medium containing 125I-LDL. The cellular association and degradation of 125I-LDL were measured according to Goldstein and Brown [15]. In experiments in which the cellular distribution of LDL receptor was studied, cytoskeletons were isolated either before or after the binding assay by exposing cells for 10 min at 0°C to 0.5% Triton X-100 in 25 mM Heps (pH 7.4) containing 1 mM PMSF [16]. The binding assay was performed by incubation of cells for 3 h at 0°C with 125I-labeled LDL (10 μg/ml).

LDL-Receptor mRNA and Protein Expression

Isolation of Total Cyttoplasmic RNA: Cells were grown to confluency in medium supplemented with FCS. Forty-eight hours prior to the experiment the cells were refed with medium containing 5% FCS or 5% LPDS. For total cytoplasmic RNA isolation a modification of the method described by Brawerman et al [17] was used [20].

S1-Nuclease Analysis: Using plasmid pLDR-2H11, kindly provided by Professor D. W. Russell [3], containing nucleotides 1572–3485 of the LDL-receptor cDNA sequence in pSP64 vector, a specific S1-nuclease analysis probe was isolated after digestion with restriction endonuclease Hinf 1. The probe contained nucleotides 3240–3485 of LDL-receptor cDNA and about 200 nucleotides of vector nucleotides After 3'end labeling (using 32P-ATP and Klenow enzyme), the probe was purified by 5% PAGE followed by electroelution (Biotrap, Schleicher & Schuell) from the gel. The hybridization of the S1-nuclease analysis was performed at 57°C [20].

Protein Isolation and Blotting Procedures: For isolation of cellular proteins a modification of the method described by Daniel et al [20] was used. Blotting procedures, including gel electrophoresis, electroblotting, and ligand blotting with 125I-labeled β-VLDL, were done, as described elsewhere [19].

Densitometric Analysis: The intensity of the bands obtained after both the S1-nuclease analysis and the ligand blotting experiments were quantified photodensitometrically (Desaga CD60).

Incorporation of 14C-Acetate into Lipids Confluent cultures were incubated for 30 h in culture media supplemented with 5% lipoprotein-deficient serum (LPDS) [21]. For measurement of the

Figure 1. The domain structure of the LDL receptor. The binding site contains six of seven cysteine-rich repeats. All six repeats (depicted as open and solid boxes, respectively) are essential for LDL binding. Only repeats depicted as solid boxes are required for binding of β-VLDL.
Figure 2. Cell association, degradation of LDL, and effect of LDL on de novo cholesterol synthesis in normal human keratinocytes cultured either at low or normal calcium concentration. (A) Differentiation-induced modulation of LDL-receptor binding (B). (A) Confluent cultures of keratinocytes grown either at low or normal calcium concentration were preincubated for 24 h in medium containing LPDS. The confluent cultures of low-calcium cells were, after preincubation in LPDS, exposed for varying time intervals to normal calcium concentration. The labeled-LDL binding, degradation, and the effect of LDL on de novo cholesterol synthesis have been assessed as described in Materials and Methods. The results on sterologenesis are expressed as percent of inhibition of de novo cholesterol synthesis by 10 μg LDL/ml.

Figure 3. Effect of extracellular calcium concentration on cell association, degradation of LDL, and effect of LDL on de novo cholesterol synthesis in normal human keratinocytes. Keratinocytes were grown to confluence at 0.06, 0.1, 0.14, 0.2, 0.3, and 1.6 mM calcium concentration and subsequently preincubated for 24 h in medium containing LPDS. The labeled-LDL binding, degradation, and the effect of LDL on de novo cholesterol synthesis have been assessed as described in Materials and Methods. The results on cholesterol synthesis are expressed as percent of inhibition of de novo cholesterol synthesis by 10 μg LDL/ml.

keratinocytes can be manipulated experimentally by changing the extracellular calcium concentration. When cultured under low-calcium (<0.1 mM) conditions, normal human keratinocytes form only a monolayer and exhibit a low capacity to differentiate. In contrast, at physiologic calcium concentrations (≥1.0 mM), the competence of normal keratinocytes to differentiate is markedly increased and the cells form a multilayer consisting of basal and of 2-5 suprabasal layers [23].

The studies with normal human keratinocytes revealed that LDL receptors are expressed in keratinocytes grown under differentiation-retarding conditions; i.e., in low-calcium media. Under these conditions, LDL is taken up by the cells where it is degraded, and de novo cholesterol synthesis is inhibited (Fig 2A). In contrast, in cultures grown at physiologic calcium concentration, the sterologenesis is not significantly affected by LDL due to the extremely low LDL-receptor activity (Fig 2A). Induction of keratinocyte differen-

Figure 4. Differentiation-linked change in the expression of LDL receptor at membrane, mRNA, and protein levels in normal human keratinocytes cultured at low (0.06 mM) (open box) and normal (1.6 mM) (solid box) calcium levels. The results are normalized, taking the expression in cells grown in normal keratinocytes as reference.
Figure 5. Cell association, degradation of LDL, and effect of LDL on de novo cholesterol synthesis in SCC-12F2, SCC-15, and SCC-4 cells. The SCC cells were grown to confluence at normal calcium concentration and subsequently preincubated for 24 h in medium containing LPDS. The 125I-LDL binding and effect of LDL on de novo cholesterol synthesis have been assessed as described in Materials and Methods. The results on cholesterol synthesis are expressed as percent of inhibition of de novo cholesterol synthesis by 10 μg LDL/ml.

Regression by exposure of low-calcium cells to physiologic calcium concentration results in a rapid decrease in LDL binding to its receptor, resulting in the loss of LDL-dependent cholesterol synthesis (Fig 2B) [24]. As shown in Fig 3, this loss of LDL-dependent cholesterol synthesis starts already at calcium concentrations between 0.06 and 0.14 mM, at which the cells virtually lose the ability to bind and degrade LDL, resulting in independency of cholesterol biology on extracellular lipoproteins.

Differentiation-linked modulations of LDL receptor activity at the plasma membrane level are a reflection of the modulations in LDL-receptor synthesis: induction of keratinocyte differentiation is accompanied by a 2-3 times decrease in LDL-receptor mRNA expression and by about a 40 times decrease in the LDL-receptor protein expression (Fig 4) [20]. The finding that the changes in LDL-receptor expression at the level of mRNA are much more profound than those observed at the mRNA level suggests that in normal human keratinocytes the regulation of LDL receptor expression is regulated not only at the mRNA but also at the protein level.

All data presented so far concern confluent keratinocyte cultures. It should be noted, however, that the extent of differentiation of keratinocytes is not only dependent on the extracellular calcium concentration but also on changes in normal calcium medium during the culture period: it is low in sparse cultures and gradually increases, reaching a maximum in post-confluent cultures. When keratinocytes are grown at physiologic calcium concentrations they generate functional LDL receptors only at the early stages of growth, at which time the extent of keratinocyte differentiation is low; i.e., in pre-confluent cultures, and they progressively lose this property over time [25]. In confluent and post-confluent cultures, the rate of cholesterol synthesis is independent of extracellular lipoproteins [21,24] and is very high in differentiating keratinocytes, suggesting a high demand there of cholesterol [21,24,25]. Morphologic observations, in which receptor binding and internalization were studied in normal keratinocytes, have been performed to complete the information from the biochemical experiments. Namely, keratinocytes cultured at a physiologic calcium concentration showed LDL binding only in subconfluent cultures in the outermost (non-stratifying) regions of the cell colonies, whereas those cultured at a low calcium concentration showed considerable binding [24]. Furthermore, only keratinocytes grown at a low calcium concentration were able to bind and internalize LDL-gold conjugates, as revealed by ultrastructural studies [24].

Regulation of LDL Receptor Expression in Malignant Keratinocytes Relation of the LDL-receptor expression to the state of cell differentiation can be further studied using malignant keratinocyte cell lines derived from squamous cell carcinomas. These cell lines differ in the degree of differentiation disorder in the order: normal keratinocytes < SCC-12F2 < SCC-15 < SCC-4 [11,12].

Figure 6. Differentiation-induced modulation of cornified envelope formation, binding of LDL, LDL-receptor DNA, mRNA, and protein expression in normal and malignant keratinocytes. Data on keratinocyte differentiation capacity (A) were taken from Boonstra et al. [31]; data on LDL-receptor gene number (B), on the LDL-receptor mRNA expression (C) and protein expression (D), and on the 125I-LDL binding (E,F) were taken from te Pas et al. [18].
Changes in LDL-receptor distribution. In experiments in which the binding of LDL was measured in intact cells, in addition to isolated cytoskeletons, it was shown that the LDL-receptor expression at the membrane level in intact low-calcium cells is high (Fig 7). A Triton X-100 extraction following \(^{125}\text{I}\)-LDL binding resulted in an 80% decrease of LDL binding. These binding sites represent the cell-surface, cytoskeleton-associated LDL receptors. Isolation of cytoskeletons of normal human keratinocytes following \(^{125}\text{I}\)-LDL binding.

These cells, when cultured at physiologic calcium concentrations, express LDL-receptors, internalize, and degrade LDL, leading to LDL-induced reduction of cholesterol synthesis (Fig 5) [28]. The LDL-receptor binding has been found to be inversely related to the capacity of the cells to differentiate (Fig 6A) and can be regulated by extracellular calcium concentration (as in normal keratinocytes) (Fig 6B) [20]. Similarly, as with normal keratinocytes, in malignant keratinocytes both LDL receptor expression and LDL-dependent cholesterologenesis vary with cell density [27,28].

In order to elucidate how LDL-receptor expression is regulated, the LDL-receptor gene numbers were determined in normal human keratinocytes and in a variety of SCC lines. These studies revealed that the cells could be divided into two classes: i) cells with amplified LDL-receptor gene numbers, i.e., poorly differentiating SCC-4 and SCC-15 cells; and ii) cells without gene amplification, i.e., well-differentiating SCC-12F2 cells and normal keratinocytes (Fig 6C) [18]. The observed differences in gene numbers have been found to be reflected at the mRNA and protein levels. Namely, cells without LDL-receptor gene amplification (normal keratinocytes and SCC-12F2) have comparable LDL-receptor expression at the mRNA and protein levels, in contrast to cells with amplified LDL-receptor gene numbers, which exhibit enhanced LDL-receptor mRNA levels (Fig 6D,E). Suppression of differentiation of malignant keratinocytes by lowering extracellular calcium concentration causes, as in normal keratinocytes, an increase of LDL-receptor expression at the mRNA and protein levels (Fig 6D,E) [20]. These findings indicate that LDL-receptor mRNA and protein expression is dependent on the differentiation state of the cell, because it can be regulated by external calcium concentrations.

On the basis of results obtained in studies with normal and malignant keratinocytes it is concluded that the regulation of LDL-receptor expression occurs at the DNA level (gene amplification), mRNA, and protein levels, depending upon the cell type studied.

**Figure 7.** Redistribution of LDL receptors in normal keratinocytes during calcium-induced differentiation. Confluent cultures of keratinocytes were grown to confluence under low calcium conditions and subsequently exposed to normal calcium conditions for varying time intervals, after which the binding of LDL to intact cells or Triton X-100–treated cells was assessed, as described in Materials and Methods. (B) \(^{125}\text{I}\)-LDL binding to intact cells; T/B: LDL binding to isolated cytoskeletons (\(^{125}\text{I}\)-LDL binding was assessed after Triton X-100 treatment). These binding sites represent intracellular, cytoskeleton-associated receptors; B/T: a Triton X-100 extraction following \(^{125}\text{I}\)-LDL binding. These binding sites represent the cell-surface, cytoskeleton-associated LDL receptors.

**Figure 8.** Electronmicrograph showing details of suprabasal cells in normal (a) and in psoriatic (b) epidermis. Lowicryl K4M-embedded tissue sections were incubated with a monoclonal anti-LDL receptor antibody followed by a goat–anti-mouse IgG conjugated to 15 nm colloidal gold. In normal epidermis, the LDL is mainly located in the cytoplasm (arrowheads); in psoriatic epidermis most of the LDL receptors were detected at the cell surface (arrowheads). Magnification X 25,000.
resulted in a 40% decrease of LDL binding as compared to that in intact cells. Induction of differentiation by exposure of low-calcium cells to physiologic calcium concentrations resulted in a marked decrease of plasma-membrane–located LDL binding sites, accompanied by a marked increase of 125I-LDL binding to isolated cytoskeletons (Fig 7) [29], resulting in the ratio of intracellular, cytoskeleton-associated receptors to plasma-membrane–located receptors of about 4. This suggests that the cells grown at physiologic calcium concentrations contain a high number of intracellular, cytoskeleton-associated receptors. In contrast to the intracellular, cytoskeleton receptors, the fraction of cell-surface, cytoskeleton-associated receptors did not change upon calcium-induced differentiation. This finding was unique for normal keratinocytes, as this phenomenon was not seen with any of the malignant keratinocyte lines tested.

At present, the function of these receptor subpopulations is unknown. Also, the nature of the associated cytoskeletal domain is unknown. It is perhaps relevant that similar observations in the expression [30–32] and cellular distribution [29] of EGF receptors during cell differentiation have been found with both normal keratinocytes and SCC cells, suggesting that a similar mechanism may underly the regulation of the activity of various membrane receptors. Because in differentiating normal keratinocytes a high proportion of LDL and EGF receptors is localized inside the cells, the cytoskeletal association of receptors may play a regulatory role in the differentiation of normal human keratinocytes, as receptors that become inaccessible to the ligand cannot serve for transduction of extracellular stimuli and may cause the cells to differentiate.

Regulation of LDL-Receptor Expression In Vivo

Differentiation-related modulations of the LDL-receptor expression and of the cellular distribution found with cultured keratinocytes parallel observations in the epidermis. When the distribution of LDL receptors is studied in cells freshly isolated from the epidermis using LDL gold as the ultrastructural marker, abundant LDL-gold binding and uptake occur only in keratinocytes exhibiting the morphologic characteristics of basal cells and not in the suprabasal, more differentiated keratinocytes [33]. The link between the stage of epidermal differentiation and the LDL-receptor expression and cellular localization is further supported by observations made in psoriasiform epidermis in situ [22]. There, LDL receptors are not only present on basal cells but also on suprabasal cells that express markers of hyperproliferation. Thus, it appears that in the basal cells in which the LDL uptake system is predominantly operative, epidermal cholesterologenesis can be influenced by extracellular LDL-cholesterol levels, the level depending on the amount of LDL reaching this cell layer. In contrast, the sterologenesis in the postmitotic, differentiating layers would not be affected substantially. Whereas the LDL-gold technique allows visualization of LDL binding to the plasma membrane and subsequent internalization, immunoelectronmicroscopic studies with LDL-receptor antibodies provide information on the cellular localization of LDL receptors in the absence of a ligand. With this technique it has been found [22] that in basal and normal epidermis, LDL receptors are distributed evenly between the cell surface and the cytoplasm, whereas in suprabasal cells most of the LDL receptors are present inside the cells (Fig 8a). In contrast to normal epidermis, in psoriasis suprabasal cells, the LDL-receptor was located predominantly on the cell surface (Fig 8b). Similar observations as with normal human epidermis in situ have also been made recently with keratinocytes cultured at the air-liquid interface [34]; these reveal that cellular LDL-receptor distribution is not affected by extracellular lipoproteins.

One of the most active tissues in cholesterol synthesis is the epidermis. Sterologenesis in suprabasal epidermis equals or even exceeds that found in basal cell layers [35]. This is easily understood on the basis of the information presented above. Suprabasal epidermal cells possess a much lower number of active LDL receptors and must thus rely on de novo cholesterol synthesis. Epidermal cholesterol synthesis appears to be therefore relatively autonomous from systemic influences; neither dietary nor circulating sterol levels seem to affect cutaneous sterologenesis significantly [36,37]. Despite its autonomy from the circulation, epidermal sterologenesis can be regulated by cutaneous barrier requirements, i.e., disruption of the permeability barrier by solvent, detergent treatment, essential fatty deficiency, or by the application of competitive HMGCoA reductase inhibitors results in increased epidermal sterologenesis [38–42]. Sterologenesis returns to normal as barrier function normalizes [40,41] or if the barrier function is restored by the application of a water-impermeable membrane to the skin surface or by the topical application of cholesterol [40–42].

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