A mutation and an antibody that affect chemical cross-linking of low-density lipoprotein receptors on human fibroblasts

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Treatment of normal fibroblasts with the bifunctional cross-linking reagent DTSSP [3,3'-dithiobis(sulphosuccinimidylpropionate)] at 4 °C converted approximately 40 % of the cell-surface low-density lipoprotein (LDL) receptors into a high- M_r form, thought to represent receptor dimers. Preincubation of the cells with anti-(LDL receptor) monoclonal antibody 10A2 increased the proportion of surface receptors in the high- M_r form after treatment with DTSSP at 4 °C to over 70 %. Preincubation with LDL did not affect the proportion cross-linked, but prevented the increase produced by antibody 10A2. Cross-linking at 37 °C was less efficient than at 4 °C and was not affected by

preincubation with antibody 10A2. Surface LDL receptors on fibroblasts from the homozygous familial hypercholesterolaemic subject MM were not cross-linked by DTSSP, confirming that the mutation had produced a change in the conformation of the receptor molecule. Taken together, the results suggest that normal LDL receptors on at least one region of the surface membrane may be loosely associated in some form of multimeric array which alters its alignment differently in response to antibody 10A2 and to cooling. Mutations that alter the tertiary structure of the receptors could affect LDL binding by disturbing the arrangement of the array.

INTRODUCTION

The low-density lipoprotein (LDL) receptor is a glycoprotein present on the surface of most cells which mediates the uptake and degradation of LDL [1]. It is synthesized as a precursor which undergoes processing in the Golgi apparatus before being inserted in the surface membrane [2]. van Driel et al. [3] have shown that some, at least, of these receptors are close enough in the membrane to be linked by bifunctional cross-linking agents. However, it is not known if this association plays any part in determining the binding properties of the receptor. During our previous experiments we have come across two instances where LDL binding appears to have been affected by some degree of aggregation of receptors in the cell membrane. In one, a monoclonal antibody (mAb) directed against the LDL receptor, mAb 10A2, halved LDL binding and uptake, suggesting that the antibody had cross-linked two adjacent receptors [4]. In the other, cells from a homozygous familial hypercholesterolaemic subject (MM) were shown to produce a receptor that was not only slowly processed but also bound only one-half as much LDL as would be expected from the amount of receptor protein on the cell surface [5]. In the experiments reported in this paper, we have studied the effect of the bifunctional cross-linking agent DTSSP [3,3'-dithiobis(sulphosuccinimidylpropionate)] on the receptors on MM and normal cells. The results show that the extent of chemical cross-linking is affected by temperature and preincubation of the cells with mAb 10A2 and LDL. They suggest that LDL binding can be modulated by changes in the alignment of receptors on the cell surface and that it is possible for mutations in regions away from the binding site to affect binding by disrupting the normal interaction between receptors.

METHODS

Human skin fibroblasts were maintained in culture and seeded (1.2×10^6 cells/dish) into 9 cm Petri dishes as described previously [6]. They were grown for 5–7 days, until they covered the bottom of the dish and were preincubated for 40 h in medium containing

5 % lipoprotein-deficient serum (LPDS, 2.5 mg of protein/ml) before experiments.

LDL ($\rho = 1.019\text{--}1.063$ g/ml) and LPDS were prepared from fresh plasma from normal healthy donors as described before [7]. MAbs 4B3 and 10A2 were raised and screened against purified LDL receptor and were purified and labelled with Na¹²⁵I as described by Soutar and Knight [8].

Cells were preincubated for 1 h at 37 °C in medium containing 5 % LPDS with 2.67 nM mAb 10A2 and 50 μ g/ml LDL protein as required. The procedures used to treat the cells with cross-linking reagent and to solubilize the receptors were essentially those described by van Driel et al. [3]. Briefly, cells were washed twice with Hepes/NaCl buffer (50 mM Hepes, 100 mM NaCl, pH 7.4) at the appropriate temperature and incubated for 30 min at 4 °C or 37 °C in Hepes/NaCl buffer containing 1 mg/ml DTSSP (Pierce Europe BV, Oud-Beijerland, The Netherlands). The reaction was stopped by the addition of glycine (10 mM) and the cells were washed with ice-cold Hepes/NaCl buffer and harvested in Hepes/NaCl buffer containing 1 mM phenylmethanesulphonyl fluoride, 10 mM EDTA, 10 mM EGTA, 10 mM *N*-ethylmaleimide and 2.2 % (v/v) dimethyl sulphoxide. Cells treated with DTSSP were aggregated and formed clumps on being scraped from the dish. These tended to adhere to the dish, which reduced the amount of protein that could be speedily recovered. After centrifugation, the cells from two dishes were lysed in 100 μ l of the same buffer containing 1 % (w/v) Triton X-100 and 0.5 mM leupeptin. The lysate was centrifuged for 5 min in a Beckman Microfuge and 50 μ l of the supernatant was immediately subjected to SDS/PAGE in 7 % (w/v) gels under non-reducing conditions as described by Laemmli [9]. Proteins were transferred to nitrocellulose membranes and the LDL-receptor protein was detected with ¹²⁵I-labelled antibodies as described by Soutar et al. [10]. Autoradiograms were prepared on preflashed film with intensifying screens at –70 °C and scanned with a Joyce–Loebl Chromoscan III densitometer (Joyce–Loebl, Gateshead, U.K.). Under these conditions the peak area was linearly related to the amount of receptor protein

applied to the gel [10]. The protein contents of the Triton-soluble extracts were assayed by the method of Bensadoun and Weinstein [11].

RESULTS

Cultured human fibroblasts that had been preincubated with LPDS to induce the production of LDL receptors were incubated at 4 °C with the cross-linking reagent DTSSP, and the Triton-soluble proteins separated by SDS/PAGE and transferred to nitrocellulose membranes. MAb 4B3, which binds to the human LDL receptor, recognized two major bands of protein on the membrane (Figure 1, lane b). One, of M_r 130 000, corresponded to the LDL receptor monomer. The second, of M_r 225 000, was not present in the absence of DTSSP (Figure 1, lane a) and presumably represents LDL-receptor protein cross-linked to

either itself or some other membrane component. This higher- M_r species was less abundant if the cross-linking was performed at 37 °C (Figure 1, lane d). The amount of radioactive antibody bound to each band was determined by densitometric scanning of the autoradiograms. The total detected was reduced after incubation with DTSSP but this was exactly matched by the decrease in the recovery of cell protein (Table 1, see the Methods section), suggesting that each cross-linked protein retained the same number of antigenic determinants for mAb 4B3 as the monomer. Thus the distribution of antibody between the bands can be taken to reflect the distribution of the receptor protein itself. On this basis it was estimated that approx. 25 % of the receptor protein in the cells was cross-linked after treatment with DTSSP at 4 °C and 12 % after treatment at 37 °C (Table 1).

Preincubation of the cells with LDL at 37 °C did not influence the amount of receptor protein that could subsequently be cross-linked at 4 °C (Table 1). In contrast, preincubation at 37 °C with anti-receptor mAb 10A2 significantly increased the proportion in the cross-linked band to almost 40 % (Figure 1, lane c; Table 1). Interestingly this increase was prevented if LDL was also included in the preincubation medium (Figure 1, lane e; Table 1) and did not occur if the cross-linking reaction was performed at 37 °C (Table 1). The presence of LDL or mAb 10A2 during the cross-linking reaction with DTSSP at 4 °C had no effect on the amount of cross-linked receptor detected. The increase in cross-linked receptor produced by mAb 10A2 was dependent on the concentration of the antibody in the preincubation medium (Figure 2). The effect was saturable, with the maximum increase achieved with approximately 1 nM antibody and 50 % of maximum with approximately 0.1 nM antibody. These values were similar to the concentrations required for essentially maximum and half-maximum binding of the antibody to surface LDL receptors [4]. Preincubation with mAb 10A2 had no obvious effect on the mobility of the cross-linked band and did not give rise to any detectable bands of different size (Figure 1, lane f). Also, the cross-linked band did not bind anti-mouse IgG antibodies that recognized mAb 10A2. Thus there was no evidence that the DTSSP had covalently linked the antibody to the receptor protein.

Although the cross-linked receptor apparently exposed all its antigenic sites for mAb 4B3, it showed markedly decreased binding of mAb 10A2 (Figure 1, lane g). Assuming similar binding of the two antibodies to the monomer, it was estimated

Figure 1 Detection of LDL-receptor bands by immunoblotting

Cells from a normal or homozygous familial hypercholesterolaemic subject (MM) were preincubated at 37 °C with 2.67 nM mAb 10A2 (Ab) or 50 µg/ml LDL protein as indicated and then with DTSSP for 30 min at the temperature shown. Triton-soluble proteins were separated by SDS/PAGE and the LDL-receptor protein was detected with ¹²⁵I-labelled mAb 4B3 (lanes a–f, h–k) or ¹²⁵I-labelled mAb 10A2 (lane g). Sizes were estimated by comparison with standards of known size (high- M_r markers, Sigma, St. Louis, MO, U.S.A.).

Table 1 Proportion of the LDL receptor in the cross-linked band

Autoradiograms obtained as described in Figure 1 were scanned with a scanning densitometer and the areas under the cross-linked (M_r 225 000) and monomer (M_r 130 000) peaks determined. Total receptor detected was the sum of the areas for the cross-linked and monomer bands and is expressed as a percentage of that observed with cells cross-linked at 4 °C prepared at the same time and run on the same gel. The protein content of the Triton-soluble cell extract is expressed in the same way. The amount of cross-linked receptor is given as a percentage of the total, and all results are the means ± S.E.M. of the numbers of determinations shown in parentheses. *Significant ($P < 0.05$) effect on antibody 10A2 (Student's t test); †significant ($P < 0.05$) effect of DTSSP (Student's t test).

Preincubation at 37 °C	Cross-linking		Cross-linked receptor (% of total)	Total receptor (% of +DTSSP at 4 °C)	Protein (% of +DTSSP at 4 °C)
	DTSSP	Temp. (°C)			
–	–	4	2.9 ± 0.7 (6)	160 ± 11	158 ± 4
–	+	4	25.0 ± 1.2† (12)	100	100
+ LDL	+	4	25.7 ± 2.4 (5)	88 ± 6	86 ± 4
+ 10A2	+	4	38.5 ± 1.3* (9)	109 ± 9	103 ± 2
+ LDL + 10A2	+	4	24.5 ± 2.3 (5)	103 ± 9	96 ± 10
–	–	37	1.9 ± 1.0 (5)	118 ± 8	125 ± 11
–	+	37	12.9 ± 1.1† (5)	91 ± 10	89 ± 4
+ 10A2	+	37	13.6 ± 2.2 (3)	87 ± 4	94 ± 2

Figure 2 Effect of mAb 10A2 on LDL-receptor cross-linking

Cells were preincubated for 1 h with the indicated concentration of mAb 10A2. After incubation with DTSSP at 4 °C for 30 min the Triton-soluble proteins were subjected to immunoblotting as described in the Methods section and the amount of receptor detected in the cross-linked band was expressed as a percentage of the total. Each point represents the results from two dishes of cells.

that the cross-linked receptor bound 35–40% as much mAb 10A2 as mAb 4B3. This was not improved by increasing the concentration of mAb 10A2 in the detection reaction. The cross-linked band did not bind LDL or β -very-low-density lipoprotein. However, this was not necessarily a consequence of the covalent cross-linking. Treatment of cells with DTSSP abolished all saturable binding of LDL to the cell surface, indicating that the cross-linker had also reacted with essential lysine residues in the binding region.

Incubation of fibroblasts from the homozygous familial hypercholesterolaemic subject MM with DTSSP at 4 °C did not produce any high- M_r cross-linked LDL-receptor protein that could be detected with mAbs 4B3 or 10A2 (Figure 1, lane i). There was no visible band of M_r 225000, even if the gels were overloaded or the cells preincubated with mAb 10A2 or LDL. Although the amount of receptor monomer was reduced during the cross-linking reaction, this was again exactly matched by a decrease in the amount of protein extracted. Furthermore, the proportion of total receptor protein (mature plus precursor) in the mature form after incubation with DTSSP ($44 \pm 5\%$, mean $\pm$ S.E.M. of six observations) was not significantly different from that observed after incubation without cross-linker ($47 \pm 5\%$). Thus there was no evidence that the surface receptor had reacted with DTSSP to produce a cross-linked receptor that was not recognized by the antibodies used. Incubation of MM cells with DTSSP at 37 °C also failed to produce any cross-linked mature receptor, but resulted in an almost complete loss of precursor protein from the soluble cell extract (Figure 1, lane k). There was no such loss of precursor during cross-linking at 4 °C, confirming that DTSSP did not enter the cells at this temperature [12].

In an attempt to increase the proportion of cross-linked receptor in the cells, normal fibroblasts were incubated at 4 °C with DTSSP, then incubated in medium for 10 min at 37 °C before being reincubated at 4 °C with the cross-linker. In this way, recycling receptors that were trapped inside the cell during the first incubation were allowed to return to the surface and become accessible to the cross-linking reagent. Unexpectedly, this did not result in any great increase in the amount of cross-linked receptor detected. Indeed, with cells that had been preincubated with mAb 10A2, the proportion of cross-linked receptor was actually reduced. This strongly suggested that the original chemically cross-linked receptor had been cleaved during

Figure 3 Loss of cross-linked band on incubation at 37 °C

(a) Cells were preincubated for 1 h at 37 °C with (●) or without (○) 2.67 nM mAb 10A2 and treated with DTSSP at 4 °C for 30 min. These were then incubated for up to 10 min at 37 °C in culture medium containing 5% LPDS. After immunoblotting, the amount of receptor in the cross-linked band was expressed as a percentage of that in the monomer. Each point represents the results from two dishes of cells. (b) Cells were treated with DTSSP at 4 °C for 30 min and incubated for up to 10 min in culture medium (□, ■) or HEPES/NaCl buffer (△, ▲) with (closed symbols) or without (open symbols) 20 μ M dihydrocytochalasin B (Aldrich, Gillingham, Dorset, U.K.). After immunoblotting, the amount of cross-linked receptor in each experiment was expressed as a percentage of that observed with no incubation (control). Results shown are means $\pm$ S.E.M. for four experiments without dihydrocytochalasin B and the averages of two experiments with dihydrocytochalasin B.

the incubation at 37 °C. To confirm this, cells that had been preincubated with and without mAb 10A2 were incubated with DTSSP at 4 °C and then harvested after various periods of incubation in medium containing LPDS at 37 °C (Figure 3a). In both cases the amount of cross-linked receptor had noticeably decreased within 30 s and was barely detectable after 5 min. Results from four experiments (Figure 3b) showed that over 80% of the cross-linked receptor was lost after 2 min incubation in culture medium. However, the total receptor detected at this time was $98 \pm 5\%$ of that present before incubation, indicating that the cross-linked receptor had returned to its monomeric form and had not been degraded. If the cells were incubated in HEPES/NaCl buffer rather than whole culture medium, the cleavage of the cross-linked receptor was delayed but not prevented (Figure 3b). Cleavage was not affected by the presence of LPDS in either medium, nor by preincubation of the cells with a concentration of dihydrocytochalasin B that inhibited endocytosis (Figure 3b).

DISCUSSION

van Driel et al. [3] cited a variety of evidence to support their conclusion that the high- M_r LDL-receptor band produced by the action of cross-linking agents on intact cells represented receptor molecules linked as dimers. The size was the same as that produced by cross-linking pure receptors and was within the range expected for a dimer. Also, only LDL-receptor monomers were released on reduction of immunoprecipitated radioactive

complexes linked with cleavable reagents. The band that was formed in our experiments after treatment of human skin fibroblasts with DTSSP was identical in size with that observed by van Driel et al. [3], and it seems reasonable to propose, by analogy, that this also was a dimer of receptor molecules rather than a compound of receptors with some other protein. The covalent link formed by DTSSP was rapidly broken when the cells were warmed to 37 °C. The mechanism responsible for this cleavage is not clear, but is not apparently related to the process of endocytosis itself since it was not inhibited by dihydrocytochalasin B. DTSSP does not permeate through cell membranes [12] and so did not react at 4 °C with the LDL-receptor precursor protein that accumulated within MM cells. However, none of the precursor remained in the soluble cell extract after a 30 min incubation with DTSSP at 37 °C, presumably because it had become linked to some Triton-insoluble intracellular protein. The abnormal precursor in MM cells is thought to be held up in the Golgi apparatus through inefficient processing of its sugar residues, which indicates that at 37 °C the cross-linking agent can rapidly gain access to the interior of the cell, possibly in endocytic vesicles that deliver their contents near the Golgi itself.

At 4 °C, approx. 25% of the total LDL-receptor protein in normal cells was cross-linked by DTSSP alone, which represents about 40% of the receptors trapped on the cell surface [4]. This proportion increased to 70% if the cells were preincubated at 37 °C with mAb 10A2, suggesting that the antibody had joined and constrained those receptors that were otherwise not suitably aligned for cross-linking. However, the stimulation by the antibody only occurred when the cross-linking reaction with DTSSP was performed at 4 °C and not when it was performed at 37 °C. Furthermore, LDL prevented the effect of the antibody even though it does not inhibit its binding to the receptors [4]. Thus the joining of receptors by mAb 10A2 at 37 °C was not sufficient to bring them into the correct configuration to allow cross-linking by DTSSP, and there must have been some extra constraint that occurred as the cells were cooled. It is known that the binding of LDL is decreased and the affinity increased when they are cooled to 4 °C [4]. Also, the efficiency of cross-linking in the absence of antibody was much greater at 4 °C than at 37 °C (Table 1). Therefore it is probable that there is an aggregation or change in the configuration of the receptors in the membrane during cooling that modifies their binding properties and renders some of them more susceptible to cross-linking by DTSSP. Cross-linking by mAb 10A2 could promote these changes in the remaining receptors, but only if there is no interference, presumably through steric hindrance, from bound LDL.

The behaviour of MM cells supports this interpretation. The binding properties of MM cells indicate that their LDL receptors are more strongly associated than those on normal cells, effectively masking some of the binding sites for LDL [5]. However, as with the joining of normal receptors by mAb 10A2 at 37 °C, any such association did not, in itself, facilitate chemical cross-linking. Indeed, the change brought about by the mutation prevented cross-linking by DTSSP, even at 4 °C. A notable feature of these cells is that they do not demonstrate the reduction in binding or change in affinity for LDL that is observed on cooling normal cells [5]. This suggests that the mutant receptors could not undergo the normal change of configuration that occurs on cooling and, since they also were unaffected by DTSSP, supports the contention that such a rearrangement is necessary to bring the receptors together in a way that permits efficient chemical cross-linking.

In normal cells LDL blocked the effect of mAb 10A2 on cross-linking by DTSSP, yet had no effect in the absence of the

antibody. This indicates that there could be two structurally distinct pools of receptors on the cell surface, only one of which contained receptors that could achieve the correct configuration for cross-linking by DTSSP without being constrained by the antibody. Whether these represent receptors in different regions of the cell surface or different receptors in some oligomeric association cannot be deduced from these experiments. However, recent evidence from trapping experiments suggests that LDL receptors on the surface of fibroblasts are present as oligomers [13], and electron microscopy of gold-labelled cells shows groups of loosely associated receptors [14]. The results presented here are certainly consistent with the proposal that LDL receptors in at least one region of the cell surface are in a loosely associated cluster or array. Further, they indicate that the arrangement of receptors can alter as the fluidity of the membrane changes during cooling. The ability to vary the alignment of its components is an essential requirement for any array that can modulate its activity. The demonstration that LDL receptors have this ability suggests that receptor realignment could also be responsible for other changes in their properties, for instance the increase in affinity for LDL on cholesterol depletion [15], under conditions that would be expected to change the fluidity of the cell membrane.

More direct evidence that receptor configuration could have a physiological role in modulating LDL binding is provided by MM cells. Subject MM is homozygous for a mutation in the LDL-receptor gene that changes a proline residue at position 664 to a leucine [16], and results in a marked reduction in both the apparent affinity of the surface receptors for LDL and the amount of LDL bound per receptor [5]. The inability of DTSSP to cross-link the surface receptors of MM cells confirms that the mutation had produced a change in the tertiary structure of the receptor molecules which altered their alignment in the cell membrane. Since the substitution is some 300 amino acids from the proposed binding region, these observations strongly suggest that it is this disruption of alignment between receptors that is responsible for the change in binding properties in MM cells and, by implication, that some interaction between receptor molecules is necessary for the efficient binding of LDL by normal cells. They also raise the interesting possibility that other mutations distant from the binding region of the LDL receptor could affect LDL binding and so give rise to familial hypercholesterolaemia by disturbing the configuration and preventing the normal changes in alignment of receptor molecules on the cell surface.

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