Does the D-galactose receptor on Kupffer cells recycle?

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Kupffer cells carry on the cell surface a lectin-like receptor with specificity for D-galactose/D-glucose residues. After tryptic digestion of receptors, new lectin activity appears on the plasma membrane within 15 min. Protein biosynthesis *de novo* is not involved. This indicates the transfer of new lectin molecules stored within the cell to the cell surface. Kupffer cells can enter a physiological state where receptor recycling does not take place.

Macrophages have been found, in recent years, to express on the cell surface a number of different lectin-like receptors that are able to mediate endocytosis of soluble and/or particulate glycoconjugates (Kolb & Kolb-Bachofen, 1978; Weir *et al.*, 1979; Stahl *et al.*, 1980; Warr, 1980; Nagamura & Kolb, 1980). The galactose receptors on rat Kupffer cells and peritoneal macrophages have in addition been shown to mediate cell contacts with autologous D-galactose-expressing cells (Kolb *et al.*, 1979; Nagamura & Kolb, 1981). In the experiments presented here we have studied whether the galactose-specific lectin shows recycling behaviour, i.e., whether after consumption of membrane-bound receptors new lectin from an intracellular pool is integrated into the membrane. Lectin activity on the cell surface was determined by optical microscopy (binding of large particles with terminal D-galactose residues) or by electron microscopy (binding of asialo-glycoproteins coupled to an electron-dense marker). Both types of experiments indicate the transfer of new lectin molecules stored within the cell to the cell surface. In addition, we have found that, after phagocytosis of carbon particles, Kupffer cells enter a physiological state where receptor recycling does not take place, i.e. receptors mediate only once in the uptake of ligand and do not reappear on the cell surface.

Experimental

Male Wistar rats (160–200 g of body weight) were kindly provided by Professor L. Herberg (Diabetes Research Institute, University of Düsseldorf).

Kupffer cells were isolated from the rat liver by a collagenase perfusion technique described previously (Schlepper-Schäfer *et al.*, 1980). Collagenase was

purchased from Boehringer (Mannheim, Germany). The presence of D-galactose receptors on the cell surface was demonstrated by the ability of Kupffer cells to bind spontaneously at 4°C erythrocytes expressing terminal D-galactose residues (neuraminidase-treated rat erythrocytes) (Kolb & Kolb-Bachofen, 1978; Kolb *et al.*, 1979; Schlepper-Schäfer *et al.*, 1980). Freshly isolated Kupffer cells (10^5 cells in 50 μ l of Eagle's medium) were mixed with an equal volume of neuraminidase-treated erythrocytes (5×10^6 cells). Cells were co-centrifuged, incubated for 1 h at 4°C, resuspended and cell aggregates observed in a haemocytometer. Binding was regarded as positive when three or more asialo-erythrocytes adhered to a single Kupffer cell. By this criterion more than 90% of liver macrophages show lectin activity. This type of cell interaction is Ca^{2+} -dependent and can be specifically inhibited by saccharides related in structure to D-galactose and by glycoproteins with terminal D-galactose or D-glucose residues (Schlepper-Schäfer *et al.*, 1980; Kolb *et al.*, 1980).

Carbohydrate inhibition experiments were performed by preincubation of Kupffer cells with saccharides (Sigma, Munich, Germany) for 5 min at 0°C.

Phagocytosis was induced by injecting rats intravenously with 0.06 ml of Indian ink (Pelikan Fount India; Gunther Wagner, Hannover, Germany)/100 g body weight, which had been dialysed against 0.9% NaCl (Schlepper-Schäfer *et al.*, 1980). Kupffer cells were isolated after 30 min.

For electron microscopy, asialofetuin (Bauer *et al.*, 1976) was coupled to colloidal gold as described by Horrisberger & Rosset (1977). The average diameter of the gold particles was 17 nm. Isolated Kupffer cells were incubated with gold-asialofetuin

for 20 min at 37°C and processed for electron microscopy as described previously (Schlepper-Schäfer *et al.*, 1980).

Results and discussion

Freshly isolated rat Kupffer cells express on the cell surface a lectin-like receptor for D-galactose/D-glucose residues that is able to mediate cell contacts with desialylated rat erythrocytes. Fig. 1 shows that trypsin treatment of Kupffer cells at 4°C destroys binding capacity for desialylated cells. The number of Kupffer cells positive in the cell-adhesion

Fig. 1. Re-expression of Kupffer-cell lectin activity after trypsin treatment.

Lectin activity was determined by the ability of Kupffer cells to bind desialylated (D-galactose-residue-expressing) rat erythrocytes. 'Untreated': freshly isolated Kupffer cells were incubated with desialylated rat erythrocytes and the number of macrophages binding three or more erythrocytes was determined. 'Trypsin-treated (1c)': Kupffer cells were incubated with trypsin (2 mg/ml) for 5 min at 0°C, washed three times with Eagle's medium + 10% (v/v) foetal-calf serum and subsequently tested for residual lectin activity in the erythrocyte-binding assay. 'After 15 min, 37°C (1c)': after trypsin treatment and washing, cells were incubated for 15 min at 37°C before the binding assay. In a parallel experiment, Kupffer cells were kept in the presence of cycloheximide (50 µM) throughout the whole procedure. This concentration of cycloheximide had been shown previously to block protein biosynthesis in rat macrophages (Stahl *et al.*, 1980). 'Trypsin-treated (2c)': second cycle of trypsin treatment. 'After 15 min, 37°C (2c)': second cycle of warming cells to 37°C. 'Sham-treated': same treatment of cells, except that trypsin was omitted.

test decreases from above 90% to 20%. Binding capacity, however, is fully recovered by briefly warming cells to 37°C. Newly expressed receptors were inactivated by a second cycle of trypsin treatment and the cells again regained their previous binding capacity after 15 min of incubation at 37°C. The same result was seen in the presence of cycloheximide, thus synthesis of receptors *de novo* is not involved (Fig. 1). The reappearance of cell-adhesion activity is due to the re-expression of lectin molecules, as shown in Fig. 2. After trypsin treatment and recovery, cell contacts have the same carbohydrate specificity as before. In both cases binding between cells is blocked by D-galactose and N-acetyl-D-galactosamine, but not, or only slightly, by D-mannose and N-acetyl-D-glucosamine. The same specificity pattern has been described previously for the D-galactose/D-glucose-binding lectin on Kupffer cells (Schlepper-Schäfer *et al.*, 1980).

Tryptic digestion was also performed with Kupffer cells that had undergone 'unspecific' phagocytosis of carbon particles. As shown in Fig. 3, such cells lose their lectin activity after trypsin treatment, but do not recover binding capacity after warming cells to 37°C. The warming period could be extended up to 1 h without any apparent re-expression of lectin molecules on the cell surface. Thus, after phagocytosis of carbon particles, the mechanism of galactose receptor reappearance in the Kupffer cell membrane is blocked.

The finding of two different physiological states of

Fig. 2. Carbohydrate specificity of cell contacts between Kupffer cells and desialylated erythrocytes

After trypsin treatment and warming to 37°C for 15 min, Kupffer cells were tested for binding activity in the presence of various monosaccharides. At 25 mM, N-acetyl-D-galactosamine (GalNAc) and D-galactose (Gal) strongly inhibit cell contacts, whereas D-mannose (Man) and N-acetyl-D-glucosamine (GlcNAc) did not.

EXPLANATION OF PLATE 1

Ultrastructure of lectin-mediated endocytosis of gold-asialofetuin by Kupffer cells

(a) Continuous uptake of asialofetuin by naïve Kupffer cells. Kupffer cells were incubated with gold-asialofetuin for 20 min at 37°C. A representative section of a Kupffer cell demonstrating the endocytosis of large amounts of gold-asialofetuin particles (large arrowheads) and the existence of lectin-binding sites on the cell surface (small arrowheads), indicating continuous uptake of ligand, are shown. n, nucleus. (b) Lack of receptor re-expression in Kupffer cells after phagocytosis of carbon particles. Kupffer cells that had undergone phagocytosis (see the legend to Fig. 3) were incubated with gold-asialofetuin particles as described above. A representative section of a Kupffer cell is shown, demonstrating that after the endocytosis of a small amount of gold-asialofetuin particles (large arrowheads) no further lectin-binding sites appear on the cell surface. Asterisks mark ingested carbon particles; n, nucleus; the bar represents 1 μ m.

Fig. 3. D-Galactose receptor re-expression is blocked in Kupffer cells that have undergone phagocytosis of carbon particles

Kupffer cells were isolated 30 min after phagocytosis of carbon particles and tested for lectin activity in the erythrocyte-binding assay. The experimental procedure was the same as described in the legend to Fig. 1.

Kupffer cells with respect to their D-galactose receptor recycling behaviour is confirmed by electron-microscopic observations (Plate 1). In these experiments, membrane-bound lectins were made visible by their binding of asialofetuin [a glycoprotein with a triantennary complex oligosaccharide with terminal D-galactose residues (Bauer *et al.*, 1976)] adsorbed to gold particles as electron-dense marker (gold-asialofetuin).

Plate 1(a) shows a thin section through a Kupffer cell that had been incubated with excess gold-asialofetuin particles for 20 min at 37°C. It is apparent that the cell has endocytosed massive amounts of gold-asialofetuin particles and binds additional particles to the cell surface. Binding and uptake is D-galactose-specific-lectin-mediated, since it is inhibited by more than 90% in the presence of 25 mM-N-acetyl-D-galactosamine but not by N-acetyl-D-glucosamine or by D-mannose.

Plate 1(b) shows a comparable section through a Kupffer cell that has undergone phagocytosis of carbon particles and was subsequently incubated with gold-asialofetuin particles for 20 min at 37°C as above. The Kupffer cell has taken up a small amount of gold-asialofetuin particles; no binding activity is left on the cell surface. Initial binding of ligand at 4°C and uptake of receptor-ligand complexes within 5 min at 37°C was found to be the same as in naïve Kupffer cells. But, whereas in naïve

Kupffer cells ligand binding and uptake is a continuous process leading to the accumulation of gold-asialofetuin particles within the cell (see Plate 1a), it appears that, in Kupffer cells that have undergone phagocytosis of carbon particles, receptors mediate the uptake of ligand only once and are not recycled to the cell surface. Thus electron-microscopic observations confirm the above findings that the mechanism for receptor re-expression is impaired after phagocytosis of carbon particles.

In conclusion, the experiments reported here show that D-galactose-specific lectins on the Kupffer-cell surface can be rapidly replaced after their tryptic inactivation without any protein biosynthesis involved. The transfer of new receptors to the cell surface, however, is affected by the physiological state of the cell; it is blocked in macrophages that have previously phagocytosed carbon particles. New receptors probably stem from an intracellular pool, although the existence of a trypsin-resistant and inactive form of the receptor on the plasma membrane cannot be excluded. Such a membrane pool, however, would have to be rather large in order to allow receptor re-expression twice (after two cycles of trypsin treatment; see Fig. 1).

It is noteworthy that receptor recycling and an internal pool of receptors have been suggested for two other lectins involved in receptor-mediated endocytosis, the D-mannose-specific lectin in rat alveolar macrophages (Stahl *et al.*, 1980) and the D-galactose-specific lectin on hepatocytes (Tanabe *et al.*, 1979). In the latter case it was found that after receptor inactivation by neuraminidase treatment or specific-antibody binding, new lectin activity did not appear on the cell surface (Stockert *et al.*, 1980a,b). This does not imply, however, that in hepatocytes membrane-bound and intracellular receptors cannot be exchanged, since we have shown here that the mechanisms of membrane receptor replacement may depend on the physiological state of cell.

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