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Effect of temperature on endocytosis and degradation of sulphated proteoglycans by cultured skin fibroblasts

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Abstract. Temperature up to 16°C reduced endocytosis of [^{35}S]-proteoglycans by human skin fibroblasts to less than 15% of that at 37°C. At temperatures between 20-26°C endocytosis was more than 50%. At temperatures below 26°C, the relative rate of degradation of endocytosed [^{35}S]-proteoglycans was several fold less than the rate of endocytosis.

Codistribution of endocytosed [^{35}S]-proteoglycans and the lysosomal marker enzyme β-hexosaminidase upon subcellular fractionation indicated that endocytotic vesicles containing [^{35}S]-proteoglycans had fused with lysosomes at 37°C and at 16°C. The prolonged halflives of endocytosed [^{35}S]-proteoglycans at 16-26°C could not be explained merely by a temperature dependent reduction of catalytic activity of lysosomal enzymes participating in the degradation of sulphated proteoglycans.

Keywords. Proteoglycan endocytosis; lysosomes; reduced degradation.

Introduction

Cells maintained in culture synthesize sulphated proteoglycans and distribute them in varying amounts into extracellular, cell surface associated and intracellular pools (Neufeld and Cantz, 1973; Kresse *et al.,* 1975). Previous studies have shown that arterial and skin fibroblasts internalize sulphated proteoglycans from the exterior through a process of adsorptive endocytosis involving specific binding of these macromolecules to cell surface receptors (Kresse *et al.,* 1975; Prinz *et al.,* 1978; Truppe and Kresse, 1978). The endocytotic vesicles so formed fuse with the lysosomes where the sulphated proteoglycans are rapidly degraded. Pastan and Willingham (1981) have recently described the formation of a new short lived subcellular organelle called "receptosome" which selectively delivers macromolecules entering cells via receptor mediated endocytosis through clathrin coated areas of the cell membrane (coated pits) to intracellular orgnelles such as lysosomes and the Golgi-endoplasmic-reticulum-lysosome (GERL) (Pastan and

Abbreviations used: GERL, Golgi-endoplasmic-reticulum-lysosome; MEM, Eagle's minimum essential medium.

Willingham, 1981). It is not known at present whether sulphated proteoglycans are internalized via receptosomes. Absorptive endocytosis is inhibited at low temperature. This may result from deficiency of ATP (Jacques, 1969), inhibition of the recycling of receptors (Weigel, 1981) or impaired fusion of endocytotic vesicles with lysosomes (Dunn *et al.,* 1980).

In our earlier studies (Prinz *et al.,* 1978), the kinetics of proteoglycan binding to cell surface suggested the presence of high affinity binding sites on cell surface and the protein core of the proteoglycan was found to be essential for binding to cell surface. As an extension of these studies, the effect of temperature on the binding, internalization and degradation of the sulphated proteoglycans by human skin fibroblasts was examined.

Materials and methods

Sodium [^{35}S]-sulphate, carrier free (5 Ci/mg S) was obtained from Amersham Buchler, Braunschweig, Germany. Sephadex G-200 and Percoll were obtained from Pharmacia, Uppsala, Sweden.

Cell culture

Human skin fibroblasts from healthy individuals were maintained at 37°C in 5% CO_2 in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (Boehringer Mannheim, Mannheim, Germany), antibiotics and non-essential amino acids as described (Cantz *et al.,* 1972).

Preparation of [^{35}S] -proteoglycan from fibroblast secretions

Confluent cultures of human skin fibroblasts in 75 cm^2 Falcon plastic flasks were incubated in the presence of 15 ml serum free medium containing 7 μCi/ml [^{35}S]-sulphate for 5 days and [^{35}S] -proteoglycan secreted into the medium were purified by gel filtration as described earlier (Prinz *et al.,* 1978). The [^{35}S]-proteoglycans had a specific activity of 15×10^6 cpm/μmol uronic acid.

Measurement of [^{35}S] -proteoglycan endocytosis

Fibroblasts grown to confluency in 21 cm^2 Falcon plastic dishes were incubated in 21 cm^2 Falcon dishes in 1.5 ml medium containing [^{35}S]-proteoglycans at a concentration of 7.5 nmol disaccharides/ml. The dishes were maintained at different temperatures in air tight boxes under a gas phase of 5% CO_2 in air for 24 h. Cells were harvested by trypsinisation at the respective temperatures. Endocytosis, adsorption and degradation were determined earlier (Kresse *et al.,* 1975; Prinz *et al.,* 1978). The rate of endocytosis and degradation was independent of the amount of [^{35}S]-proteoglycans added as its concentration was very high.

Subcellular fractionation over Percoll

Confluent monolayer cultures in 75 cm^2 Flasks were maintained in 7 ml medium containing 7.5 nmol disaccharide/ml of [^{35}S] -proteoglycan for 24 h. Cells were harvested by trypsinisation at 16°C. All subsequent operations were done at 4°C. After washing with saline and then with MEM containing 10% calf serum, the cell pellet was washed with 0.25 M sucrose, resuspended in 0.25 M sucrose, kept for 10 min under nitrogen at 10 atm and homogenized with a glass homogenizer. The

homogenate was centrifuged at 600 *g* for 10 min at 4°C. The postnuclear fraction was applied on a Percoll gradient (starting density 1.07 g/ml) centrifuged in a vertical rotor VTi50 (Beckman Instruments) at 20000g for 1 hat 4°C (Pertoft *et al.,* 1978). Fractions of 2 ml were collected from the bottom by filling from the top with paraffin oil. In each fraction, density (Jurd and Rickwood, 1978), activity of β-hexosaminidase and radioactivity were determined.

Determination of half life of endocytosed [^{35}S] -proteoglycans

The rate of degradation of endocytosed [^{35}S]-proteoglycan was determined by measuring the rate of release of alcohol soluble radioactivity in a medium containing no radioactive material.

Other methods: β-Hexosaminidase, β-glucuronidase, α-N-acetyl-glycosaminidase and arylsulphatase B were determined as described (Von Figura,1977). Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Radioactivity was measured in a Packard liquid scintillation spectrometer model 2450 B using Instagel (Packard Instruments) as scintillation medium.

Results and discussion

Effect of temperature on the endocytosis of [^{35}S] -proteoglycan

Below 16°C uptake was less than 10% of that at 37°C whereas at 20-26°C, 40-50% residual uptake was observed (figure 1). Between 16-26°C the rate of degradation, however, was 3-14% of that at 37°C. Thus in this range of temperature uptake was

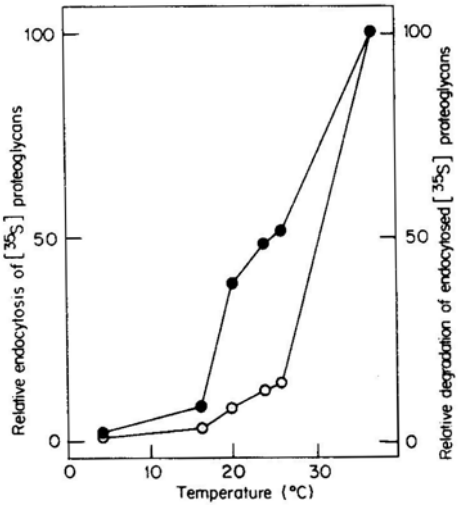

Figure 1. Endocytosis (=) and degradation (o) of [$_{35}$S] -proteoglycans by human skin fibroblasts as a function of temperature.

[^{35}S]-Proteoglycan endocytosis and degradation of endocytosed [^{35}S]-proteoglycans were determined after incubation of fibroblasts in the presence of [^{35}S]- proteoglycans for 24 h at temperatures between 16°C and 37°C. Endocytosis and degradation are expressed as % of the values at 37°C. At 37°C 12.8% of added [^{35}S] –proteoglycans were endocytosed and 65% of the endocytosed material had become degraded during the 24 h incubation period.

affected less than degradation of sulphated proteoglycans. The amount of proeoglycans absorbed to the cell surface measured as the amount released by trypsin was not affected by varying the temperature, It was 10% less at 16°C than at 37°C (data not given).

Subcellular fractionation after endocytosis at 16°C

A reduced rate of degradation at 16-26°C could result either from impairment of the fusion of endocytotic vesicles with lysosomes and/or from a decrease of the lysosomal activity at 16-26°C.

To decide whether the retarded rate of degradation was due to the inaccessibility of the endocytosed material to the lysosomes at this lower temperature, fibroblasts were incubated in the presence of [^{35}S]-proteoglycans at 16°C for 24 h and were

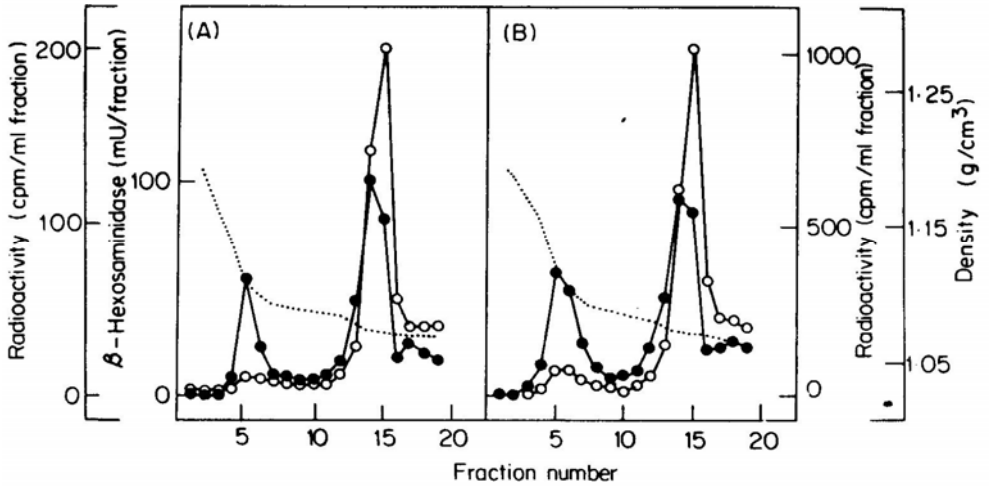

Figure 2. Subcellular fractionation on Percoll.

Postnuclear supernatant of skin fibroblasts which had been incubated in the presence of [^{35}S] - proteoglycans for 24 h at 16°C (A) or for another 160 min at 37°C (B) were fractioned on an isoosmotic gradient of Percoll. [^{35}S]-Radioactivity (●) β- hexosaminidase activity (O) and density (……) were determined in each fraction.

fractionated on an isoosmatic density gradient made of percoll. The cells incubated with [^{35}S]-proteoglycans for 24 h at 16°C and then for 160 min at 37°C to allow for transfer of internalized material into lysosomes served, as a control. A similar distribution pattern of [^{35}S]-glycosaminoglycans and β-hexosaminidase activity which served as a marker for lysosomes was obtained on fractionating the postnuclear supernatant over Percoll (figure 2) in both cases. The relative distribution of [^{35}S]-glycosaminoglycans and β-hexosaminidase among the earlier reported two classes of lysosomes, a dense fraction and a lighter density fraction (for discussions of these two classes of lysosomes see ref. Rome *et al.* (1979) was similar. The presence of [^{35}S]-glycosaminoglycans in fractions rich in lysosomes indicates that internalized proteoglycans do reach lysosomes at temperatures of 16-26°C. This conclusion is based on the assumption that fusion of endocytotic vesicles containing proteoglycan does not take place during the isolation and fractionation procedure. In hepatocytes, where endocytotic vesicles containing

asialofetuin do not fuse with lysosomes at 20°C or below (Dunn *et al.,* 1980), the endocytotic vesicles were stable.

Half life of endocytosed [^{35}S] proteoglycan at low temperature

The endocytosed material was found to be degraded to inorganic $^{35}SO_4$ with a half life of 25 h at 37°C, whereas at 16°C it was degraded with a half life of 180 h (figure 3). At 16°C the rate of degradation is therefore about 7-fold reduced. Thus

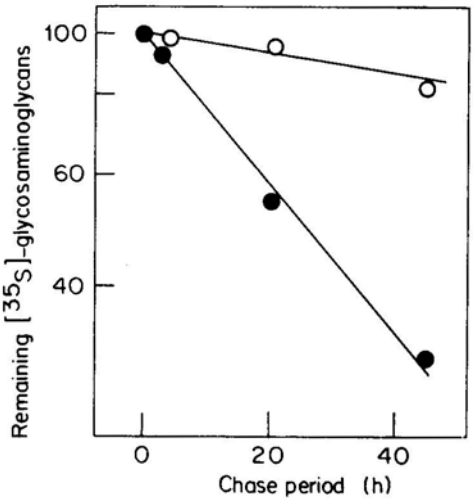

Figure 3. Half life of intracellular [^{35}S] -proteoglycans.

Fibroblasts were incubated in the presence of [^{35}S]-proteoglycans at 37°C (●) and at 16°C (O) .After 24 h the medium was removed, the cell layer washed 5 times with proteoglycan free medium and subjected to chase for upto 42 h. The degradation of [^{35}S] -proteoglycans during the chase period was determined as described in "Materials and methods".

the relative accumulation of endocytosed proteoglycans at temperatures between 16-26°C appears to result from a decreased capacity of lysosomes to degrade proteoglycans at low temperature. The levels of lysosomal enzymes involved in degradation of proteoglycans, β-glucuronidase, α-N-acetyl-glucosaminidase, β-hexosaminidase and arylsulphatase B were not affected by growing fibroblasts for 24 h at 16°C. When catalytic activity of these enzymes were measured at 37, 26 and 16°C, respectively, a reduction of 50-60% in activity was observed at 26°C and compared to 37°C and of 70-85% at 16°C.

Our results show that sulphated proteoglycans endocytosed at low temperature accumulate intracellularly. In contrast to hepatocytes, the accumulation of endocytosed material is not due to impaired fusion of endocytotic vesicles with lysosomes, but to impaired degradation intralysosomally. The decreased rate of degradation at lower temperatures, notably at 26°C cannot fully be explained by the effect of temperature on the catalytic activity of degradative enzymes. The effect of temperature on the rate of degradation of proteoglycans in intact cells may also be related to the recent findings that degradation of proteoglycans in intact lysosomes is stimulated by ATP and acetyl-CoA (Rome and Crain, 1981).

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