Synthesis and shedding of hyaluronan from plasma membranes of human fibroblasts and metastatic and non-metastatic melanoma cells

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The regulation of hyaluronan synthesis and shedding was analysed in human fibroblasts and in two melanoma cells that differed in the metastatic potential and proteolysis of the hyaluronan receptor CD44. Dissociation of nascent hyaluronan from plasma membranes isolated from fibroblasts by high salt concentrations led to activation of hyaluronan synthase. Hyaluronan synthesis was also enhanced in plasma membranes from fibroblasts that had been treated with hyaluronidase or trypsin. Hyaluronan oligosaccharides stimulated hyaluronan production

in fibroblast cultures. These results indicated that nascent high-molecular-mass hyaluronan inhibited its own chain elongation, if it was retained in the vicinity of the synthase by cell-surface receptors. The results also indicated that increased hyaluronan synthesis and shedding correlated with proteolysis of CD44 on the melanoma cell lines, which has been observed by others.

Key words: CD44, cell-surface receptors, metastasis.

INTRODUCTION

Hyaluronan is a large glycosaminoglycan that is widely sequestered in the extracellular matrix and which is secreted by many cells. Hyaluronan synthesis differs from other polysaccharides in many aspects. It is elongated by alternate transfer of UDP-hyaluronan to the substrates UDP-GlcNAc and UDP-GlcA, liberating the UDP moiety [1,2]. In contrast to other extracellular polysaccharides, hyaluronan is not synthesized in the Golgi but at plasma membranes, and nascent chains are directly extruded into the extracellular matrix [3]. Hydration and swelling of nascent hyaluronan occurs at the site of synthesis on the cell surface. It forms a coat on the cell surface that is lost upon transformation [4]. Increased hyaluronan synthesis and shedding is associated with cell migration [5,6], mitosis [7] and tumour invasion [8]. Hyaluronan is retained on the membrane-bound synthase complex during elongation and can be released from the cell surface by two mechanisms. It can dissociate as an intact molecule, or it can be degraded by hyaluronidases or radical cleavage [9]. We have shown recently that nascent hyaluronan is retained on the surface of group C Streptococci by a cell surface hyaluronan-binding ectoprotein kinase, and that loss of hyaluronan from the receptor leads to replacement by activation of the synthase [10]. In the present work we investigated whether a similar mechanism operates also in eukaryotic cell lines. For this investigation human fibroblasts, metastasizing melanoma cells MV3 and the non-metastasizing counterpart IF6, that differed in shedding of CD44 and hyaluronan synthesis, were used [11]. CD44 is the principal eukaryotic hyaluronan receptor which occurs in many isoforms and plays a critical role in the formation of several metastasizing tumour cells (for reviews see [12–14]). Our results indicated that proteolysis of CD44 led to hyaluronan shedding and activation of the synthase.

EXPERIMENTAL

Materials

Healon® was a gift from Amersham Pharmacia Biotech, radiochemicals were purchased from Amersham Pharmacia Biotech,

Biogel P-10 was from Bio-Rad, and other chemicals were from Sigma Chemical Co.

Cells and cell culture

Human melanoma cell lines MV3 and IF6 [15,16] were kindly provided by Dr. G. Van Muijen (Department of Pathology, Academisch Ziekenhuis, University of Nijmegen, The Netherlands). These cells differ in their capacity to form metastases after subcutaneous inoculation into nude mice. These cells and human fibroblasts were grown in suspension culture in Dulbecco's modified Eagle's medium supplemented with streptomycin/penicillin (100 units of each/ml), kanamycin (100 units/ml) and 10% (v/v) foetal calf serum.

Isolation of plasma membranes

Cells from 10 culture flasks (180 cm² surface area) were washed with PBS, harvested by treatment with trypsin or with the aid of a rubber policeman, sedimented at 1500 g for 5 min and suspended in 30 ml of ice-cold PBS containing 0.3 µM aprotinin, 1 µM leupeptin and 0.4 mM Pefabloc. The cells were transferred into a Parr bomb, exposed to a nitrogen pressure of 6210 kPa (900 lb/in²) for 15 min and disrupted by nitrogen cavitation [17]. Nuclei were sedimented at 2000 g for 10 min and the particulate fraction was obtained by centrifugation at 40000 g for 20 min.

Plasma membranes were isolated by the phase separation method of Brunette and Till [18]. The two aqueous phases were prepared by dissolving 40 g of Dextran T500 in 160 ml of water and 31 g of poly(ethylene glycol) 6000 in 72 ml of water. These solutions were mixed, and 333 ml of 0.22 M sodium phosphate buffer, pH 6.5, and 80 ml of water were added. The mixture was shaken in a separatory funnel and left for phase separation at 4 °C overnight. Both phases were stored separately at 4 °C. The sediment of the particulate cell fraction was suspended in 15 ml of the upper phase with the aid of a glass homogenizer and the whole was mixed with 15 ml of the lower phase. The mixture was shaken vigorously and left for phase separation in an ice bath overnight. Plasma membranes were enriched at the phase interface. They were withdrawn using a Pasteur pipette, diluted with 30 ml of cold PBS or PBS containing various concentrations

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of NaCl and sedimented at 40000 *g* for 10 min. The sediment, normally containing 3 mg of plasma membranes, was suspended in 2 ml of water with a glass homogenizer. The solution was stored at -20°C . The plasma membranes were usually enriched five-fold with respect to the specific activity of 5'-nucleotidase.

Measurement of hyaluronan synthesis and shedding

Plasma membranes (50 μg) were mixed with an equal volume of hyaluronan synthesis substrate (8 μM UDP-[^{14}C]UDP-GlcA, 166 μM UDP-GlcNAc, 4 mM dithiothreitol, 20 mM MgCl_2 , 50 mM Tris/malonate, pH 7.0) and incubated at 37°C for various time periods. The plasma membrane and the soluble fractions were separated by centrifugation for 5 min at 14000 *g* in an Eppendorf centrifuge. Hyaluronan synthesis was stopped by the addition of 10% (w/v) SDS to a final concentration of 1% of the soluble fraction, and by dissolving the plasma membrane fraction in 1% (w/v) SDS. The plasma membrane and the soluble fractions were separated by descending paper chromatography developed with ethanol/aq. 1 M ammonium acetate, pH 5.5 (13:7, v/v). After 18 h the radioactivity of [^{14}C]hyaluronan at the origin was determined.

Preparation of hyaluronan oligosaccharides

Hyaluronan oligosaccharides were prepared according to Bertrand and Delpech [19] with slight modifications. A solution of 20 ml Healon[®] containing 380 mg hyaluronan was mixed with 2 ml of 2 M sodium acetate, pH 5.0, and 50 mg bovine testicular hyaluronidase and incubated at 37°C overnight. The solution was applied to a Biogel P-10 column (143 cm $\times$ 3.6 cm) equilibrated with 10 mM NH_4HCO_3 and fractions of 14 ml were collected. Total GlcA content of the fractions was determined using the carbazole method [20]. The size of fluorescently labelled oligosaccharides in the fractions was determined by PAGE [21]. Briefly, aliquots (20 μl) were dried in a Speedvac and dissolved in 5 μl of a solution containing 64 mg/ml 8-aminonaphthalene-1,3,6-trisulphonic acid in acetic acid/water (3:17) and 5 μl of a fresh solution of 63 mg/ml sodium cyanoborohydride in DMSO. The samples were incubated at 37°C for 16 h, dried in a Speedvac and the residue was dissolved in 20 μl glycerol. The samples were separated by PAGE (30% gel) and fluorescent oligosaccharides were visualized under UV light. Fractions containing hexa- to deca-saccharides were collected and freeze dried.

Determination of hyaluronan synthesis in cell culture

The total amount of hyaluronan on fibroblasts was determined as described by Stern and Stern [22]. The effect of hyaluronan oligosaccharides on hyaluronan synthesis was determined by the measurement of [^3H]glucosamine incorporation. Human fibroblasts were seeded at a density of 4×10^4 cells/cm² on 57-cm² culture dishes in 10 ml of medium containing 0.5 mCi [^3H]glucosamine (specific activity 18.5 Ci/mmol) in the presence or absence of 50 $\mu\text{g}/\text{ml}$ of hyaluronan oligosaccharides. After incubation for 2 days the medium was withdrawn and centrifuged for 10 min at 1500 *g*. The supernatant was mixed with 3 vol. of 1.3% (w/v) potassium acetate in 97% ethanol in order to precipitate proteins and glucosaminoglycans. The mixture was centrifuged for 15 min at 4000 *g* and the sediment was suspended in 5 ml of 50 mM Tris/HCl, pH 7.8, containing 10 mg of proteases from *Streptomyces griseus*. After incubation at 37°C overnight, the solution was applied to a DEAE-Sephacel ion exchange column (5 cm $\times$ 1 cm) and eluted with 80 ml of a linear gradient of 0–0.5 M NaCl in 50 mM Tris/HCl, pH 8.4. Fractions (2 ml) were collected and the radioactivity was determined. The

salt concentration in the fractions was measured by conductometry. Authentic hyaluronan eluted at a salt concentration of approx. 0.25 M.

RESULTS

Activation of the synthase after dissociation of nascent hyaluronan in plasma membranes

Plasma membranes were isolated from human fibroblasts and incubated with the radioactive substrates for hyaluronan synthesis UDP-GlcNAc and UDP-[^{14}C]GlcA for 2 h. During this time the membranes incorporated 12000 c.p.m. [^{14}C]hyaluronan/mg of protein into the membrane fraction. The membranes were then treated with increasing NaCl concentrations and the radioactivity of [^{14}C]hyaluronan in the membrane supernatant was determined. Figure 1 shows that [^{14}C]hyaluronan was dissociated from membranes by increasing NaCl concentrations. Approx. 85% of [^{14}C]hyaluronan was liberated by 3 M NaCl. The membrane sediments were again incubated with the radioactive substrates for 2 h and the incorporation of radioactivity into total hyaluronan (e.g. into the membrane and soluble fraction) was again determined. Figure 1 shows that the total net synthesis increased with salt concentration.

The mechanism of this activation was further analysed by the kinetics of hyaluronan synthesis and shedding in isolated membranes. Figure 2(a) shows that plasma membranes incorporated hyaluronan first into the membrane fraction and then shed it into the soluble fraction. After about 1 h the synthesis ceased although the substrates were not consumed, but shedding from membranes continued for more than 8 h. When membranes were washed with 2 M NaCl to remove endogenous unlabelled hyaluronan, more [^{14}C]hyaluronan was incorporated into the membrane and soluble fractions, total net synthesis proceeded for more than 8 h and during this time the radioactivity of membrane-bound hyaluronan remained constant (Figure 2b).

Figure 1 Activation of hyaluronan synthase after dissociation of nascent hyaluronan

Plasma membranes were isolated from human fibroblasts and incubated with radioactive substrates for hyaluronan synthesis. Plasma membranes were extracted with increasing NaCl concentrations and the radioactivity of [^{14}C]hyaluronan in the extracts was determined (□). The plasma membrane fractions were again incubated with radioactive substrates for 1 h at 37°C and the total radioactivity of [^{14}C]hyaluronan was determined (■). The error bars indicate the range of two independent experiments. The variability between paired samples was significant ($P < 0.001$).

Figure 2 Kinetics of hyaluronan synthesis and release from human fibroblast membranes

Plasma membranes were isolated from human fibroblasts and the incorporation of [^{14}C]hyaluronan into the membranes (■) and in the supernatant (□) was determined at the time points indicated (a). Incorporation into NaCl-treated membranes (b), into plasma membranes isolated from hyaluronidase-treated fibroblasts (c), and into plasma membranes from trypsin-treated fibroblasts (d). The error bars indicate the range of values of two independent experiments. The variability between paired samples was significant ($P < 0.001$).

These results suggested that the synthase replaced lost hyaluronan chains.

Activation of the synthase after hyaluronidase or trypsin treatment of intact fibroblasts

It has been shown previously that treatment of intact fibroblasts with hyaluronidase led to activation of hyaluronan synthase [23]. This phenomenon was analysed in detail by the kinetics of hyaluronan synthesis and shedding in plasma membranes isolated from fibroblasts which had been treated with hyaluronidase to remove endogenous hyaluronan (Figure 2c). In comparison with the untreated control (Figure 2a), hyaluronidase treatment also led to an increase in membrane bound [^{14}C]hyaluronan that was subsequently released into the supernatant. This result indicated that large hyaluronan chains inhibited their own elongation.

If cell surface receptors were responsible for retention of hyaluronan synthesis, removal of cell surface proteins by proteases should likewise activate the synthase. When intact fibroblasts were treated with trypsin to detach them from the culture dish, the amount of cell-associated hyaluronan decreased from $3.5 \mu\text{g}/10^6$ cells to $1.4 \mu\text{g}/10^6$ cells. Plasma membranes isolated from trypsin-treated fibroblasts incorporated more hyaluronan into the membrane and soluble fraction (Figure 2d). Therefore the integrity of cell surface proteins appeared to be important for retention of nascent hyaluronan in the vicinity of the synthase and to block dissociation and exert feedback inhibition on chain elongation.

Hyaluronan oligosaccharides induced hyaluronan production in intact fibroblasts

If cell surface receptors were involved in regulating hyaluronan production, exogenously added hyaluronan oligosaccharides

Figure 3 Effect of hyaluronan oligosaccharides on hyaluronan synthesis

Human fibroblasts were labelled with [^3H]glucosamine in the absence (□) or presence (■) of $50 \mu\text{g}/\text{ml}$ hyaluronan oligosaccharides. [^3H]hyaluronan was isolated from the culture medium by DEAE-Sephacel ion-exchange chromatography and the salt concentration (●) and radioactivity were determined. The radioactivity incorporated into [^3H]hyaluronan was calculated from the area under the curves to be 1.4×10^4 c.p.m. in the absence of oligosaccharides, and 2.7×10^4 c.p.m. in the presence of oligosaccharides.

should displace nascent hyaluronan from the receptors and stimulate hyaluronan synthesis. This was demonstrated by adding a mixture of hyaluronan hexa- to deca-saccharides to human fibroblasts cultures. The oligosaccharides were obtained by hyaluronidase digestion of highly purified hyaluronan and isolation by gel filtration (results not shown). The fibroblasts were labelled with [^3H]glucosamine for 24 h and [^3H]hyaluronan was isolated from the culture medium by ion-exchange chromatography and quantified by scintillation spectroscopy (Figure 3). From these data it was calculated that $50 \mu\text{g}/\text{ml}$ of oligosaccharides stimulated hyaluronan secretion into the medium by 93 %.

Kinetics of hyaluronan synthesis and shedding from plasma membranes of metastasizing and non-metastasizing melanoma cells

The kinetics of hyaluronan synthesis in plasma membranes isolated from metastasizing melanoma cells MV3 and the non-metastasizing counterparts IF6, which differed in shedding of CD44 and hyaluronan synthesis, have been investigated previously [11]. In the present study, plasma membranes were isolated from both melanoma cell lines and were analysed for the presence of the hyaluronan receptors CD44 and RHAMM by Western blotting. While antibodies against CD44 showed prominent staining at 85 kDa, antibodies against RHAMM did not detect a protein (results not shown). In the non-metastasizing melanoma cells IF6, little radioactivity of [^{14}C]hyaluronan was incorporated into plasma membranes and more radioactivity was shed into the supernatant (Figure 4a). Whereas the incorporation into the plasma membrane fraction proceeded for more than 2 h, shedding ceased after 30 min. In metastatic cells MV3, [^{14}C]hyaluronan synthesis was enhanced and proceeded also for more than 2 h (Figure 4b) but, in contrast to the non-metastatic IF6 cells, MV3 incorporated hyaluronan mostly into the plasma membrane fraction. This situation was reversed in salt-extracted plasma membranes. In non-metastasizing IF6 cells, the release of hyaluronan into the supernatant ceased after 15 min, and incorporation into the plasma membrane fraction was enhanced when compared with untreated plasma membranes (Figure 4c). In NaCl-treated plasma membranes from metastatic

Figure 4 Kinetics of hyaluronan synthesis and shedding from plasma membranes of human melanoma cells

Plasma membranes from the non-metastatic melanoma cell line IF6 (**a, c**) and the metastatic cell line MV3 (**b, d**) were isolated and the kinetics of hyaluronan synthesis and shedding were determined as described above. The incorporation of [^{14}C]hyaluronan into the plasma membranes (■) and the plasma membrane supernatant (□) was determined at the time points indicated. For comparison, the plasma membranes were washed with 2 M NaCl before the determination of hyaluronan synthesis and shedding (**c, d**). The error bars indicate the range of values from two independent experiments. The variability for paired samples was significant ($P < 0.001$).

cells MV3, the synthesis proceeded again for more than 2 h; however, hyaluronan was now directly released into the supernatant (Figure 4d).

DISCUSSION

We have shown previously that hyaluronan synthesis in eukaryotic cells could be stimulated at several levels of intracellular signalling, including transcription and phosphorylation by cAMP- or protein-kinase-C-mediated activation [24]. In the present work we have demonstrated that hyaluronan production was also regulated on the extracellular surface. Hyaluronan is synthesized at plasma membranes and directly transferred to the cell surface [3]. The synthase elongated hyaluronan chains by 60 disaccharide units per min and produced a molecular mass of about 1.4×10^6 Da/h [1]. This was the final size of completed hyaluronan found on most cells in tissue culture [9]. When nascent hyaluronan was dissociated from isolated plasma membranes by salt treatment, the synthase was activated. Apparently, NaCl treatment liberated endogenous hyaluronan, which was replaced during subsequent synthesis *in vitro*. Kinetic analysis of hyaluronan synthesis and shedding showed that hyaluronan was first incorporated into membranes and then released slowly into the soluble fraction. Because synthesis stopped after 1 h, high-molecular-mass hyaluronan must inhibit its own elongation when bound to the synthase. Salt treatment of plasma membranes dissociated nascent hyaluronan and activated the synthase by initiating new chains. Activation of the synthase in intact cells has been observed previously following degradation of cellular hyaluronan with hyaluronidase [23] or by oxygen radicals [9]. The kinetics of synthesis and release in plasma membranes from hyaluronidase-treated cells indicated that hyaluronan synthesis

proceeded to form full-sized fragments. Apparently, endogenous high-molecular-mass hyaluronan exerted a feedback inhibition on its own chain elongation.

Trypsin treatment of intact cells activated the synthase, suggesting that hyaluronan-binding proteins could inhibit hyaluronan shedding and, thereby, initiation of new chains. Hyaluronan oligosaccharides also stimulated hyaluronan production in cell cultures, probably by displacing endogenous hyaluronan from receptors. We could detect only CD44 and not RHAMM on isolated human fibroblast plasma membranes. The role of CD44 in hyaluronan synthesis shedding was analysed in detail in metastatic and non-metastatic melanoma cell lines, which differed in the degradation of CD44 and hyaluronan production observed by others [11,25]. The non-metastatic melanoma cell line IF6 did not significantly degrade CD44, whereas the metastatic cell line MV3 produced a soluble 65 kDa fragment. The kinetics of hyaluronan synthesis and shedding in isolated plasma membranes from normal human fibroblast hyaluronan or from non-metastatic melanoma cells were substantially different from those of metastatic cells. Total net synthesis of hyaluronan was higher in plasma membranes from the metastatic cell line. Salt treatment of membranes from the non-metastatic IF6 cells led to an increase in hyaluronan incorporation into the membrane fraction, indicating that the salt-extracted chains were replaced by subsequent synthesis *in vitro*. In contrast, much [^{14}C]hyaluronan was incorporated into membranes of the metastatic MV3 cells. Apparently these membranes had already lost most of the endogenous hyaluronan which resulted in activation of the synthase and incorporation of hyaluronan into the membrane fraction. Therefore there must be some hyaluronan binding proteins on the cell surface. However, these binding proteins could be extracted by salt resulting in direct shedding of hyaluronan synthesized *in vitro* into the soluble fraction. This observation could be explained by the presence of loosely attached soluble CD44 fragments that were removed by salt treatment.

The kinetics of hyaluronan synthesis of the non-metastatic melanoma cell line IF6 was also different from those of human fibroblasts. Whereas total net synthesis in human fibroblasts stopped after about 30 min, it proceeded in IF6 membranes for more than 2 h. However, shedding of hyaluronan from fibroblasts membranes continued for more than 8 h but stopped after 15 min in IF6 membranes. These observations indicated that additional unknown factors may regulate hyaluronan synthesis and shedding.

In conclusion, the results indicated that increased hyaluronan synthesis and shedding correlated with proteolysis of CD44 on the melanoma cell lines, as observed by others, and suggested that intact cell surface CD44 may retain hyaluronan in the vicinity of the synthase to inhibit shedding and initiation of new chains. The binding of hyaluronan to cells was also influenced by the level of CD44 expression [26,27] and by CD44 modifications, such as expression of variants in different cell types [28], glycosylation [29], intracellular binding to ankyrin [30] or phosphorylation [31]. Treatment of cells with PMA, calcium and forskolin stimulated phosphorylation of CD44, reduced the hyaluronan binding activity [32] and stimulated hyaluronan synthesis [24]. All these factors may be involved in the regulation of hyaluronan synthesis and exert profound effects on migration, growth and metastasis. Reduced hyaluronan binding to CD44 could have two consequences: loss of signal transduction from the extracellular hyaluronan to intracellular phosphorylation by the CD44-associated kinase [33,34] and overproduction of hyaluronan, which expands to an enormous extent on the cell surface to cause displacement of cellular adhesion molecules.

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