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# Hyaluronan matrices in pathobiological processes

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## Abstract

Hyaluronan matrices are ubiquitous in normal and pathological biological processes. This remarkable diversity is related to their unique mechanism of synthesis by hyaluronan synthases. These enzymes are normally activated in the plasma membrane and utilize cytosolic substrates directly to form these large polyanionic glycosaminoglycans, which are extruded directly into the extracellular space. The extracellular matrices that are formed interact with cell surface receptors, notably CD44, that often dictate the biological processes, as described in the accompanying minireviews of this series. This article focuses on the discovery in recent studies that many cell stress responses initiate the synthesis of a monocyte-adhesive hyaluronan extracellular matrix, which forms a central focus for subsequent inflammatory processes that are modulated by the dialogue between the matrix and the inflammatory cells. The mechanisms involve active hyaluronan synthases at the cell membrane when cell stresses occur at physiological levels of glucose. However, dividing cells at hyperglycemic levels of glucose initiate the synthesis of hyaluronan in intracellular compartments, which induces endoplasmic reticulum stress and autophagy, processes that probably contribute greatly to diabetic pathologies.

### Keywords

autophagy; CD44; diabetes; diabetic nephropathy; endoplasmic reticulum stress; golgi; hyaluronan; hyaluronan synthase proteoglycan synthesis; inflammation

## Mechanism of hyaluronan synthesis

Hyaluronan (HA) is a glycosaminoglycan that is synthesized by a distinctly different mechanism from the other glycosaminoglycans (chondroitin sulfate, heparan sulfate, keratan sulfate). A diagram showing the mechanism of HA synthesis is given in Fig. 1. Hyaluronan synthase (HAS) enzymes are synthesized in the endoplasmic reticulum (ER) in an inactive form and must be transported in vesicles to and through the Golgi for insertion into the plasma membrane. After the enzyme has been activated, it utilizes the cytosolic substrates, UDP-glucuronate (UDP-glcUA) and UDP-*N*-acetylglucosamine (UDP-glcNAc), and adds them alternately to the reducing end of the chain with release of the anchoring UDP. The elongating chain is extruded into the extracellular compartment. Confocal microscopy



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images of live cells that were transfected with green fluorescent protein (GFP)-HAS3 are shown in Fig. 1 [1]. The localization of the enzyme (green) in perinuclear regions (ER / Golgi) and in transport vesicles is apparent. The active enzyme in the plasma membrane (yellow) extrudes HA into the normal extracellular fuzzy coats (red) with which monocytes do not interact [2] (see accompanying article by Tammi *et al.* [3]).

This mechanism of HA synthesis has several unique features [4]: (a) the extruded chain is not modified by the addition of sulfoesters or epimerases that modify other glycosaminoglycans; (b) the final chain can be extremely large, > 10 million Da; (c) a core protein is not required, unlike all proteoglycans; (d) the rate of synthesis can be modulated as a function of the concentrations of the cytosolic UDP-sugar substrates; (e) it is energetically efficient; UDP-glcUA is synthesized by two oxidation steps from UDP-glucose yielding two molecules of NADPH. It is also important to prevent the activation of HAS enzymes in intracellular compartments, which causes pathological consequences as described below.

In contrast with HA, all other glycosaminoglycans are synthesized on core proteins inside the Golgi to form the large family of proteoglycans (Fig. 2). The UDP-sugar and phosphoadenosinephosphosulfate substrates are synthesized in the cytoplasm and shuttled into the Golgi by antiporters that remove a downstream product (UMP, AMP) for each substrate, which is used to synthesize the oligosaccharide attachment region, to add the alternating sugar residues onto the nonreducing end of the growing chain and to add sulfoesters. This antiporter mechanism controls the concentrations of UDP-sugar substrates in the Golgi according to the rate of glycosaminoglycan synthesis on the proteoglycans, and is therefore independent of the changes in the UDP-sugar concentrations in the cytosol.

## Monocyte-adhesive HA matrices synthesized by stressed cells in normal glucose

Biology has taken advantage of the unique mechanism of HA synthesis to produce normal pericellular glycocalyces on most cells and to contribute to normal extracellular matrices. Notably, in cartilage, HA anchors the aggrecan proteoglycan aggregates, and this HA–aggrecan complex provides the tissue with its ability to respond to compressive loads. However, biology has also utilized the synthesis of HA to form abnormal matrices when cells are stressed by a variety of conditions. This was initially shown in a study with cultures of smooth muscle cells isolated from normal human colons [5,6]. Cultures stressed by viral infection or by treatment with poly(I:C), which initiates responses similar to viral infection, synthesized an extensive HA matrix with structural information that was recognized by monocytes / macrophages, which bind at 4 °C and rapidly phagocytose the matrix at a physiological temperature of 37 °C (Fig. 3) [6]. An increasing number of studies have now demonstrated that the same or similar monocyte-adhesive HA matrices are synthesized in response to a variety of stresses in cell models both *in vitro* and *in vivo*. For example, the section (Fig. 4) from a biopsy taken from an asthmatic patient during an inflammatory response shows an extensive pathological HA matrix (green) with embedded inflammatory cells exhibiting capped CD44 (red). Other examples include responses to ER stress at physiologically normal levels of glucose [7], wound healing [8–10], idiopathic pulmonary

hypertension [11], airway smooth muscle cells *in vitro* and airway interstitial cells in mouse asthma models [12–14], adipocytes in adipose tissue in a diabetic mouse model [15] and renal tubular endothelial stress [16–18]. Further, removal of this monocyte-adhesive matrix by inflammatory cells is essential and requires the cell surface HA receptor, CD44. This was demonstrated by showing that the lungs of CD44 null mice subjected to noxious bleomycin inhalation synthesized and continuously accumulated HA matrix which could not be removed by the influx of monocytes and macrophages [19], and most of the animals died. In contrast, irradiated CD44 null mice repopulated with normal bone marrow aspirates were able to generate normal monocytes and macrophages that were able to remove this matrix, with subsequent survival and restoration of normal lung function after bleomycin treatment. (For a further insight into the roles of HA interactions with CD44 and its variants, and their importance in malignancy, see the accompanying article by Misra *et al.* [20].)

## Monocyte-adhesive HA matrices synthesized by dividing cells in hyperglycemic glucose

More recently, a unique activation of HASs in intracellular compartments has been identified in cells stimulated to divide in hyperglycemic medium (25 mM glucose), typical of uncontrolled diabetes [21,22]. Mesangial cells isolated from rat kidneys were growth arrested and then stimulated to divide in hyperglycemic medium. This initiated a protein kinase C (PKC) response, which led to the activation of HASs in intracellular compartments, including, most probably, the ER, Golgi and transport vesicles. This is shown in the confocal micrographs of cells permeabilized at 16 h after the initiation of cell division in hyperglycemic medium and stained for HA (Fig. 5, left images). The resulting ER stress in this model initiated an autophagic response near the end of cell division, which involved a large upregulation of cyclin D3 and the formation of intracellular aggresomes that co-stained for HA, cyclin D3 and microtubule protein 9 light chain 3, a marker for autophagy [22,23]. This was followed by the formation of an extensive monocyte-adhesive HA matrix between and through neighboring cells after completion of the cell cycle, as shown in the confocal images of cultures 36 h after stimulation to divide in hyperglycemic medium (Fig. 5, right images). The inhibition of protein kinase C or the treatment of the cells with cyclin D3 siRNA prior to stimulation to divide prevented these responses. This mechanism occurs within the first week *in vivo* after the initiation of hyperglycemia in streptozotocin-treated rats [21,22]. Confocal analyses showed the presence of an abnormal HA matrix with embedded macrophages in sections from diabetic rat kidneys after 1 week [21,22]. Further, Fig. 6 shows that U937 monocytes adhere to glomeruli in such sections at 4 °C, and that they phagocytose HA out of the section when warmed to 37 °C.

## Intracellular HA: a new frontier in diabetes

A previous review has suggested the possibility that intracellular HA may be a new frontier for inflammatory pathologies [24]. An important experiment which formed the basis for this possibility showed that dividing aortic smooth muscle cells accumulate intracellular HA during the cell cycle, which is considered to be a potentially normal process [25]. However, the medium used in these experiments was hyperglycemic (25 mM glucose) which, according to our results with mesangial cells, would have activated HASs within the

dividing cells. In an unrelated earlier study, scratch wounds of endothelial cell cultures demonstrated that monocytes adhered to the migrating and dividing cells at the edges of the scratch wounds, but did not adhere to the adjacent nondividing cells [26]. These experiments were also performed in medium that contained a higher than normal glucose level (15 mM), which is above the levels shown to trigger HA synthesis within dividing mesangial cells [21]. This suggests that monocyte adhesion is most probably the result of the formation of a monocyte-adhesive HA matrix by the dividing cells. In a third case, 3T3-L1 cells, an accepted model for adipogenesis, were routinely stimulated to divide in a standard hyperglycemic (25 mM glucose) medium before stimulating their adipogenic responses. After cell division, the medium became extraordinarily viscous as a result of the synthesis of HA [27]. Figure 7 shows that, under the same conditions, adipogenic 3T3-L1 cells undergo autophagy (cyclin D3-stained aggresomes [22]) and produce an extensive HA matrix that is monocyte adhesive. These three culture models with distinctly different cell types indicate the likelihood that an intracellular HA stress response that drives autophagy and the formation of a monocyte-adhesive HA matrix will occur in most, if not all, cells stimulated to divide in hyperglycemic medium. Investigators should be aware of the glucose levels in experimental medium, as commonly used hyperglycemic media may induce intracellular HA responses in dividing cells in culture, which may confound the interpretation of the results.

Cytosolic UDP-sugar concentrations increase in cells in response to hyperglycemic conditions [28–30]. This led us to ask whether the intracellular HA synthesis response could be inhibited if the concentrations of UDP-sugars were diminished. As shown in Fig. 2, xylosides, which enter cells, enter the Golgi compartment and bypass the need for a core protein to stimulate chondroitin sulfate synthesis. The capacity of cells to synthesize chondroitin sulfate is usually much greater than the rate required to complete the proteoglycans. For example, 4-methylumbelliferol-xyloside increases chondroitin sulfate synthesis in airway smooth muscle cell cultures by eight- to ten-fold [31]. To accommodate this rate of synthesis, the antiporters must increase the entry of UDP-glcUA (a substrate for HA synthesis) and UDP-galNAc (derived from UDP-glcNAc, the other substrate for HA synthesis) into the Golgi, thereby depleting the cytosolic substrates. This was tested by stimulating mesangial cells to divide in hyperglycemic medium in the presence of this xyloside. As shown in Fig. 8, this successfully prevented intracellular HA synthesis, the subsequent stress response (autophagy and upregulation of cyclin D3) and the formation of a monocyte-adhesive HA matrix. This provides strong evidence that the levels of UDP-sugar substrates in the cytosol have a critical role in the intracellular HA synthesis response.

## Concluding remarks

Accumulating data and new findings presented here suggest that HA plays a key role in several pathological processes, and that at least two different mechanisms are involved: the stress responses of cells in normal glucose and the autophagy / cyclin D3 response of dividing cells in hyperglycemic glucose. It is worth noting that the formation of monocyte-adhesive HA matrices in a wide variety of cellular stress responses will play a central role in many, and probably most, pathologies currently confronting medical treatments. An understanding of their basic mechanisms of synthesis and of the responses of the

inflammatory and resident cells that interact with them is important for the design of appropriate ways to treat or prevent the pathological processes involved.

## Abbreviations

| | |
|---|---|
| **CD44** | cluster of differentiation 44 |
| **ER** | endoplasmic reticulum |
| **galNAc** | *N*-acetylgalactosamine |
| **GFP** | green fluorescent protein |
| **glcUA** | glucuronate |
| **glcNAc** | *N*-acetylglucosamine |
| **HA** | hyaluronan |
| **HAS** | hyaluronan synthase |
| **PKC** | protein kinase C |

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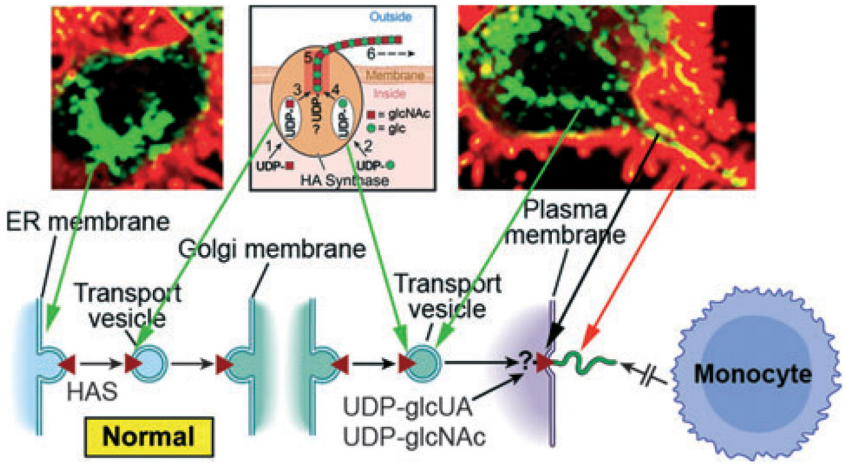


**Fig. 1.**

Model for the normal transport of hyaluronan synthase (HAS) from the endoplasmic reticulum (ER) to the plasma membrane, where it is activated to synthesize and extrude hyaluronan. The confocal micrographs show live cells that were transfected with GFP-Has3 (green) and stained for hyaluronan (red). They demonstrate ER / Golgi localization (left), transport vesicles (right), active HAS in plasma membranes (yellow) and extracellular hyaluronan (red). Micrographs provided by Kirsi Rilla (see the article by Tammi *et al.* [3] in this series).

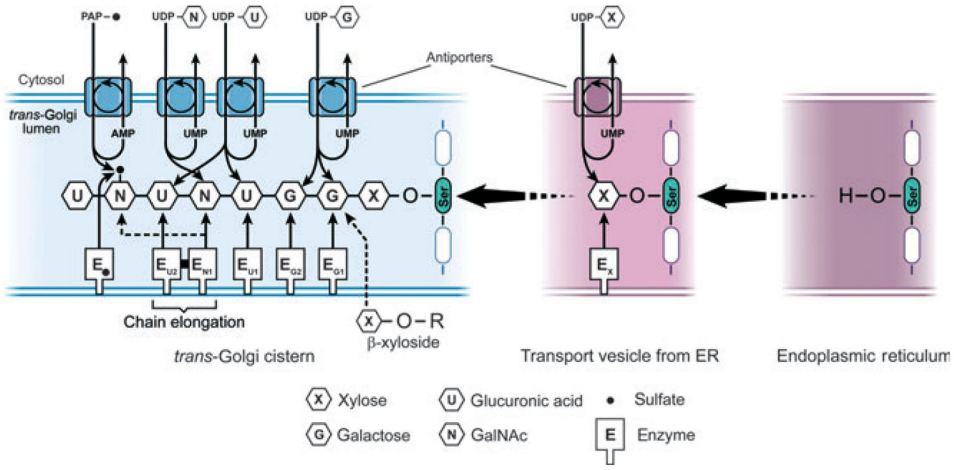


**Fig. 2.**

Model for the biosynthesis of proteoglycans (see text for details). ER, endoplasmic reticulum; PAP, phosphoadenosinephosphosulfate.

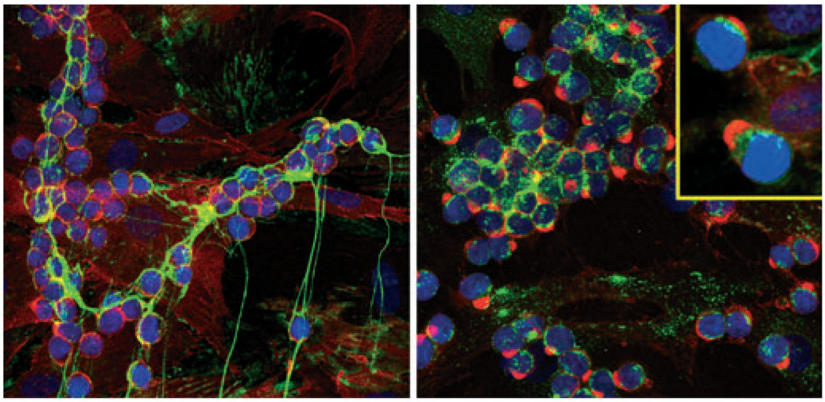

**Fig. 3.**

U937 monocytic cells, using the receptor CD44 (red), bind to hyaluronan cable structures (green) on the surface of poly(I:C)-stimulated cultures of intestinal smooth muscle cells at 4 °C (left panel) [6]. When the cultures are warmed (37 °C for 30 min), the monocytic cells relocate, or 'cap', CD44 to one pole and internalize hyaluronan as shown in the enlarged inset. The left panel is reprinted from ref. [6] with permission from the American Society for Investigative Pathology.

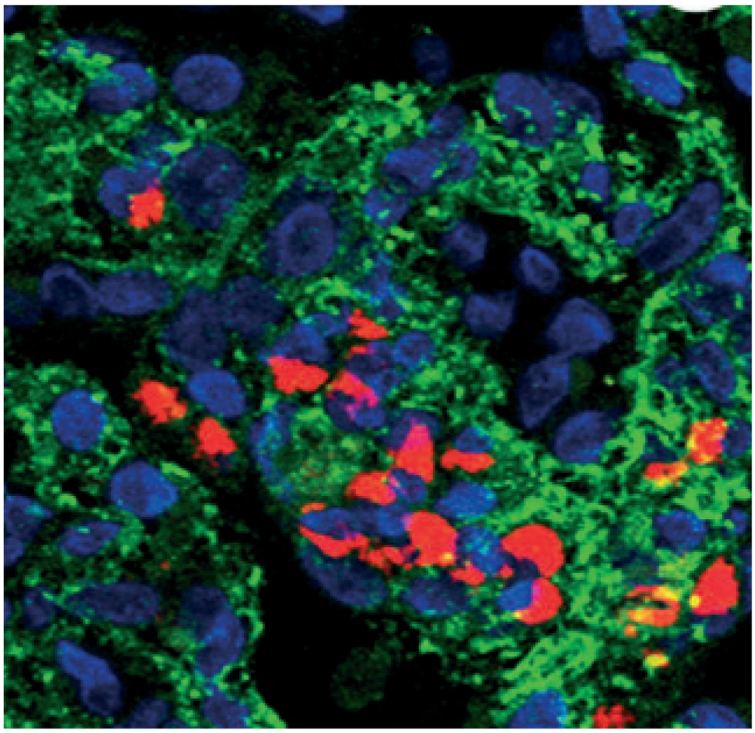

**Fig. 4.**

A section from a lung biopsy taken from a patient with an asthmatic flare stained for hyaluronan (green), CD44 (red) and nuclei (blue).

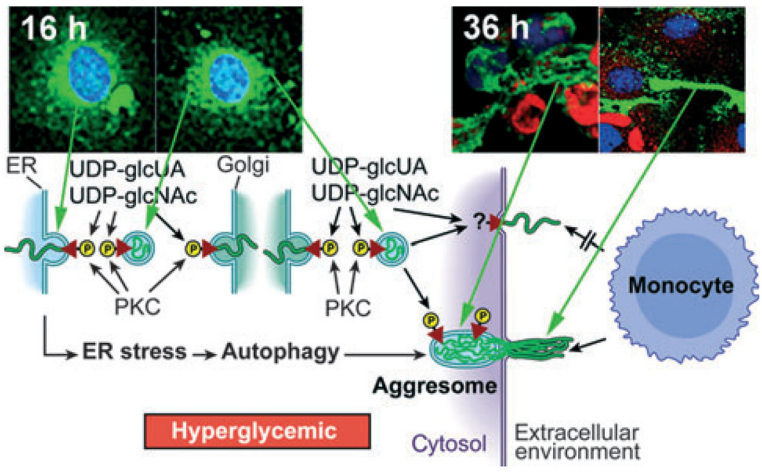


**Fig. 5.**

Model for the intracellular activation of hyaluronan synthases in cells that divide in hyperglycemic medium (25 mM glucose). The images on the left are mesangial cells stimulated to divide in hyperglycemic medium, permeabilized at 16 h and stained for hyaluronan (green). Intracellular hyaluronan is observed in endoplasmic reticulum (ER) / Golgi regions and in transport vesicles [21]. The images on the right show permeabilized cells (left) and nonpermeabilized cells (right) stained for hyaluronan (green), cyclin D3 (red) and nuclei (blue) 36 h after stimulation to divide in hyperglycemic medium [21]. PKC, protein kinase C.

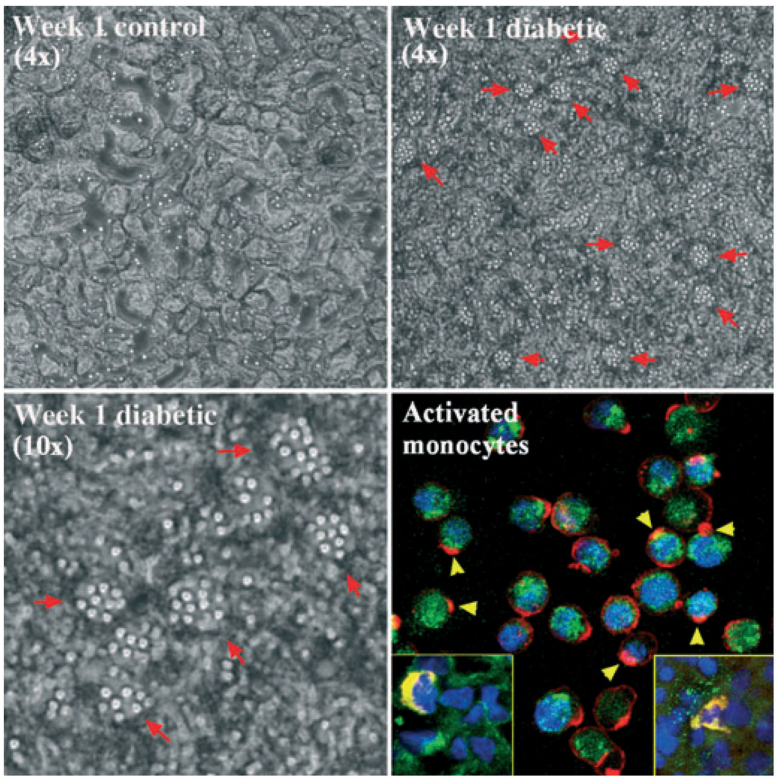


**Fig. 6.**

Adhesion of U937 monocytes to kidney sections from a control and a streptozotocin-induced diabetic rat, 1 week after the induction of hyperglycemia. An enlargement of the diabetic kidney section (bottom left) shows clusters of monocytes over glomeruli. The adhesion was performed at 4 °C. When a section from the diabetic kidney was warmed to 37 °C, most of the monocytes detached. They were then spread on a slide and stained for hyaluronan (green), CD44 (red) and nuclei (blue) (bottom right). Examples of capped CD44 are apparent (arrowheads). The insets in this panel show macrophages in glomeruli in sections that co-stain for CD44 and hyaluronan (yellow), providing evidence for monocyte / macrophage activity in the glomeruli.

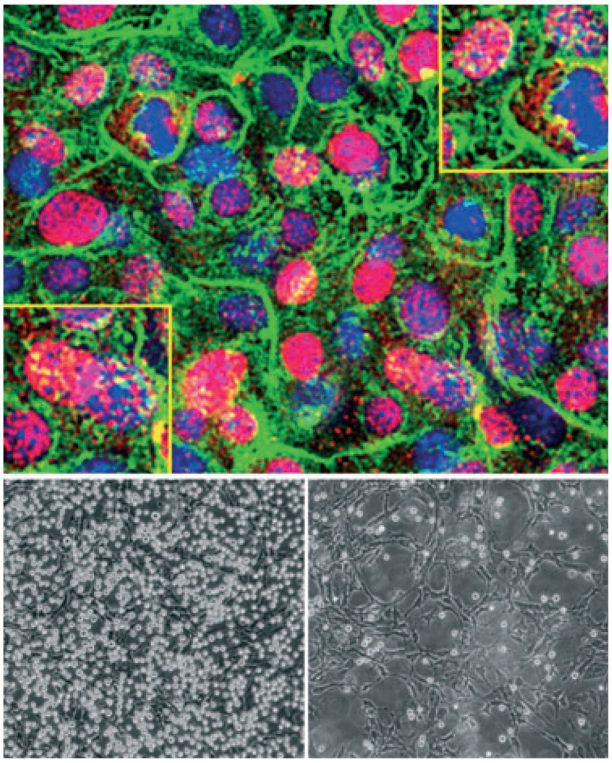

**Fig. 7.**

3T3-L1 cells dividing in hyperglycemic medium undergo autophagy and synthesize an extensive monoctye-adhesive matrix. 3T3-L1 cells were stimulated to divide in hyperglycemic medium (25 mM glucose), routinely used to promote adipogenesis in this model. At 48 h, a permeabilized culture (top panel) was stained for hyaluronan (green), cyclin D3 (red) and nuclei (blue). The presence of hyaluronan cables (green) and cyclin D3-stained aggresomes (red) indicates that the cells underwent autophagy and cyclin D3-mediated formation of a hyaluronan matrix. The bottom left panel shows extensive U937 monocyte adhesion to an identically treated culture, which was lost when the culture was treated with *Streptomyces* hyaluronidase (selective for hyaluronan) (bottom right panel).

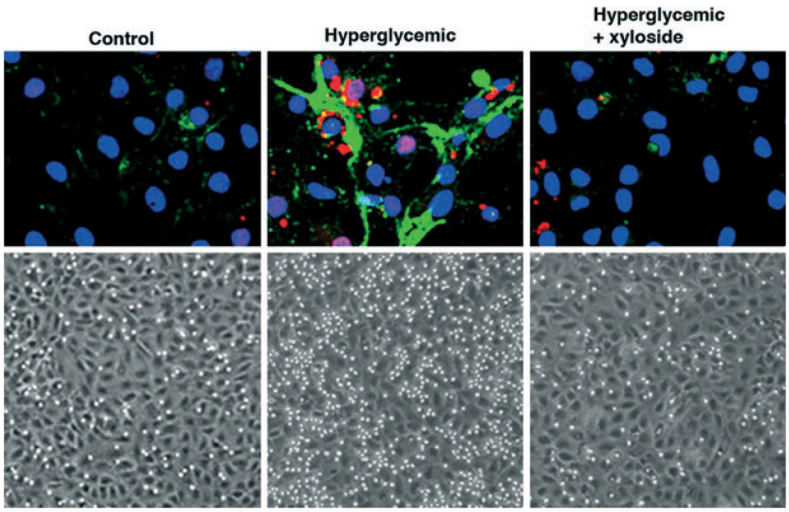


**Fig. 8.**

The treatment of mesangial cells with xyloside prevents the intracellular synthesis of hyaluronan, autophagy and the formation of a monocyte-adhesive hyaluronan matrix. Mesangial cells stimulated to divide in hyperglycemic medium produce an extensive monocyte-adhesive hyaluronan matrix and stain for cyclin D3 at 48 h (middle panels). The control (left panels) and the hyperglycemic culture treated with 0.25 mM 4-methylumbelliferone-β-xyloside do not undergo autophagy, do not synthesize intracellular hyaluronan and do not produce a monocyte-adhesive hyaluronan matrix.