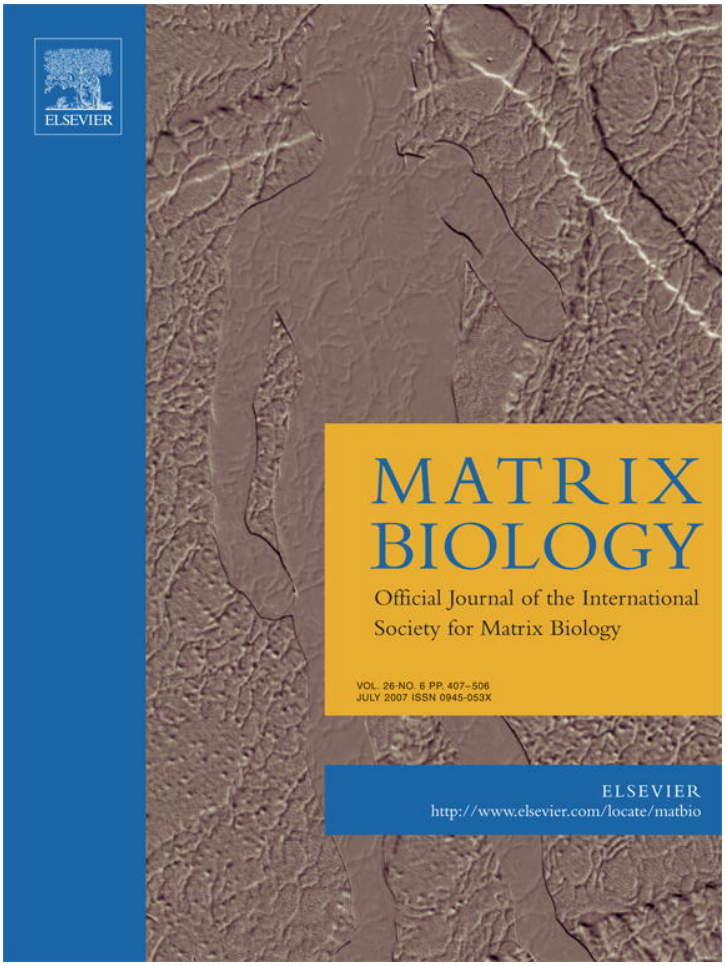

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Role of versican and hyaluronan in the differentiation of 3T3-L1 cells into preadipocytes and mature adipocytes

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Abstract

We have previously shown that during the adipose conversion of these cells the culture medium changed its viscoelastic properties due to the presence of hyaluronan and a chondroitin sulfate proteoglycan [Calvo, J.C., Rodbard, D., Katki, A., Chernick, S., and Yanagishita, M., 1991. Differentiation of 3T3-L1 preadipocytes with 3-isobutyl-1-methylxanthine and dexamethasone stimulates cell-associated and soluble chondroitin 4-sulfate proteoglycans. J. Biol. Chem. 266, 11237–11244., Calvo, J.C., Gandjbakhche, A.H., Nossal, R., Hascall, V.C., and Yanagishita, M., 1993. Rheological effects of the presence of hyaluronic acid in the extracellular media of differentiated 3T3-L1 preadipocyte cultures. Arch. Biochem. Biophys. 302, 468–475]. Here, we analyze the time course for the appearance of these molecules during drug-induced cell differentiation. The synthesis of both hyaluronan and the proteoglycan, was maximal at 48 h in the presence of isobutylmethylxanthine and dexamethasone, but while hyaluronan remained high after changing the culture medium, the proteoglycan dropped to almost basal levels after a few days. Northern analysis revealed the presence of message for a "versican-like" molecule as well as the possibility of alternative splicing. Three major bands of 9.39, 8.48, and 7.69 kb appeared in the analysis. These bands showed a dramatic increase in intensity when RNA from non-differentiated cells was compared to differentiating 3T3-L1 cells. In addition, when the time course of appearance for this message was analyzed, it perfectly correlated the metabolic labeling of the glycosaminoglycans during cell culture. The nucleotide sequencing of two exons revealed between a 100–94% homology with proteoglycan PG-M from murine endothelial cells. At least 13% of the proteoglycan was able to bind hyaluronan. Disruption of the synthesis of the proteoglycan molecule by exogenous addition of xyloside, did not prevent triglyceride accumulation but was inhibitory to preconfluent 3T3-L1 cell proliferation. Coating of plastic culture dishes with conditioned medium from differentiating 3T3-L1 cells, resulted in decreased cell adhesion. Cell adhesion was partially recovered after degradation of hyaluronan and chondroitin sulfate by enzymatic treatment. All these results indicate a possible role of these molecules in the observed changes in the viscoelastic properties of the culture medium, as well as open the field for a more thorough study of their role in 3T3-L1 cell proliferation and/or differentiation.

Keywords: Hyaluronan; Proteoglycans; Adipocytes; Differentiation

1. Introduction

For normal morphogenesis and organogenesis to occur, interactions between diverse molecules are required. These molecules include growth factors, hormones, cell adhesion molecules and extracellular matrix components. The extracellular matrix once was thought of as being a support system but now occupies a very important place in regulating size, transport, cell migration and even gene expression (Reichardt

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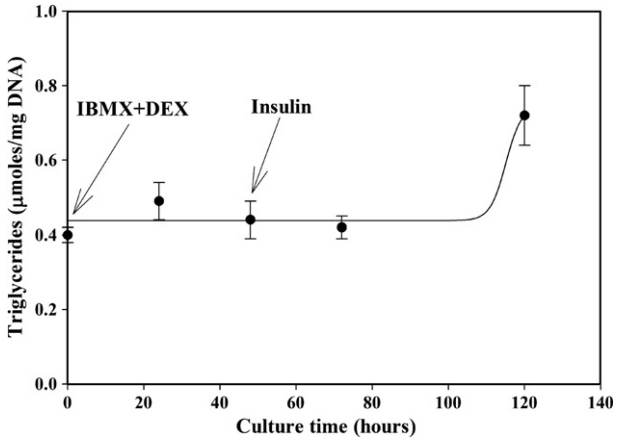

Fig. 1. Time course of lipid accumulation after differentiation of 3T3-L1 preadipocytes. 3T3-L1 cells were cultured for 48 h in the presence of dexamethasone and IBMX, 2 days after reaching a confluent state. After the differentiation period, the medium was changed to fresh culture medium containing insulin and the cells were cultured for 3 more days. This figure shows the increase in triglycerides after the addition of insulin.

and Tomaselli, 1991). This extracellular matrix includes very diverse molecules such as collagens, glycoproteins, proteoglycans and hyaluronan, among others.

Hyaluronan plays an important role as structural molecule through binding to proteoglycans, as well as influencing cell behavior, adhesion or migration through its interaction with membrane receptors, such as CD44 (Toole et al., 1977; Toole, 1990), both being strategic players in matrix assembly (Knudson, 2003). A matrix rich in hyaluronan is more fluid and allows for cell migration (Turley et al., 1985; Turley and Auersperg, 1989). The importance of hyaluronan as constituent of extracellular matrices led to the development of matrix engineering technology where reconstituted biological matrices were used to control the mechanical function of tissue (Balazs et al., 1991).

The specific interaction of hyaluronan with chondroitin sulfate proteoglycans, such as aggrecan or versican, results in aggregates with particular characteristics that contribute to the rheological properties of hyaluronan-based matrices. In cartilage, for example, the contribution of hyaluronan to matrix structure is via complexes that include the glycosaminoglycan (GAG), the proteoglycan and link protein, and provide a strongly hydrated space filling gel.

Two manuscripts by Yoneda et al. (1988a,b) present an alternate possibility for the function of hyaluronan, other than the space filling matrix. They showed that the addition of hyaluronan (HA) to confluent cultures of dermal fibroblast led to cell proliferation beyond the arrested growth. This hyaluronan was incorporated into an extracellular matrix by interaction with a chondroitin sulfate proteoglycan.

During the conversion of 3T3-L1 fibroblasts into adipocytes, the differentiation program involves early, late and very late events. Early markers (pOb24 mRNA, lipoprotein lipase) are expressed at growth arrest, whereas late and very late markers (GPDH, aP2, PEPCK and adipsin) are expressed after a limited growth resumption of preadipose cells (those cells containing early markers). The expression of early markers at growth arrest has been demonstrated by thymidine block (Amri et al., 1986b; Dani et al., 1989). After this essential step, the growth resumption of preadipose cells was shown to be critical (Kuri-Harcuch and Marsch-Moreno, 1983; Amri et al., 1986a; Ailhaud et al., 1989) and leads to terminal differentiation. It has been proposed that a combination of signals, involving cAMP, diacylglycerol and IGF-I/insulin pathways is required to trigger the mitotic activity necessary for terminal differentiation of preadipocytes (Ailhaud et al., 1989). Maximal adipocyte conversion can be attained quickly after confluence (3–5 days) by treating 3T3-L1 preadipocytes with a mixture of isobutylmethylxanthine (IBMX), dexamethasone and insulin (Calvo et al., 1991, 1993).

Although the importance of the extracellular matrix and its components on such diverse situations such as cancer invasiveness (Udabage et al., 2005), regulation of the immune system (Mummert, 2005) or tissue remodeling and differentiation is a matter of constant update, very little has been published on their role in adipose differentiation.

We have previously reported (Calvo et al., 1991) that hyaluronan and a large hydrodynamic size or “versican-like” chondroitin sulfate proteoglycan were specifically stimulated during the “chemical” differentiation of 3T3-L1 preadipocytes into adipocytes. In the present manuscript, we describe the time course of the appearance of both molecules and the identification of the large chondroitin sulfate proteoglycan as a member of the versican family.

2. Results

2.1. Triglyceride accumulation during 3T3-L1 differentiation

Fig. 1 shows the typical stimulation of triglyceride accumulation with culture time, starting on the second day after reaching confluence. The increase in triglycerides when

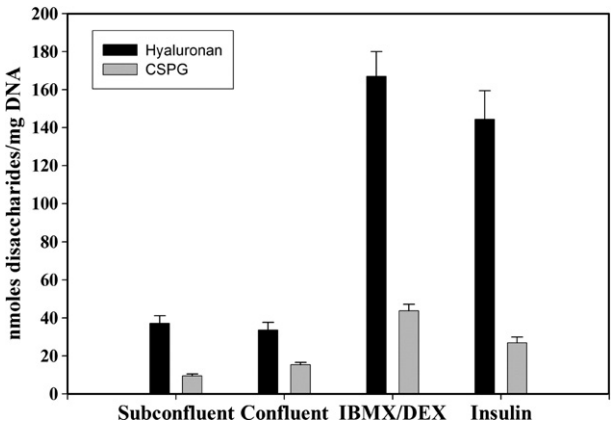

Fig. 2. Glycosaminoglycan and proteoglycan accumulation in the culture medium during 3T3-L1 differentiation. 3T3-L1 cells were metabolically labeled with [^{35}S]sulfate and [^{3}H]glucosamine and the medium was extracted for hyaluronan and proteoglycan purification. The panel shows the hyaluronan and proteoglycan accumulation in the culture medium expressed per mg of DNA. Both hyaluronan and chondroitin sulfate proteoglycans are stimulated during the dexamethasone / IBMX treatment and either remains increased (hyaluronan) or decreased slightly (proteoglycans) after the medium was changed.

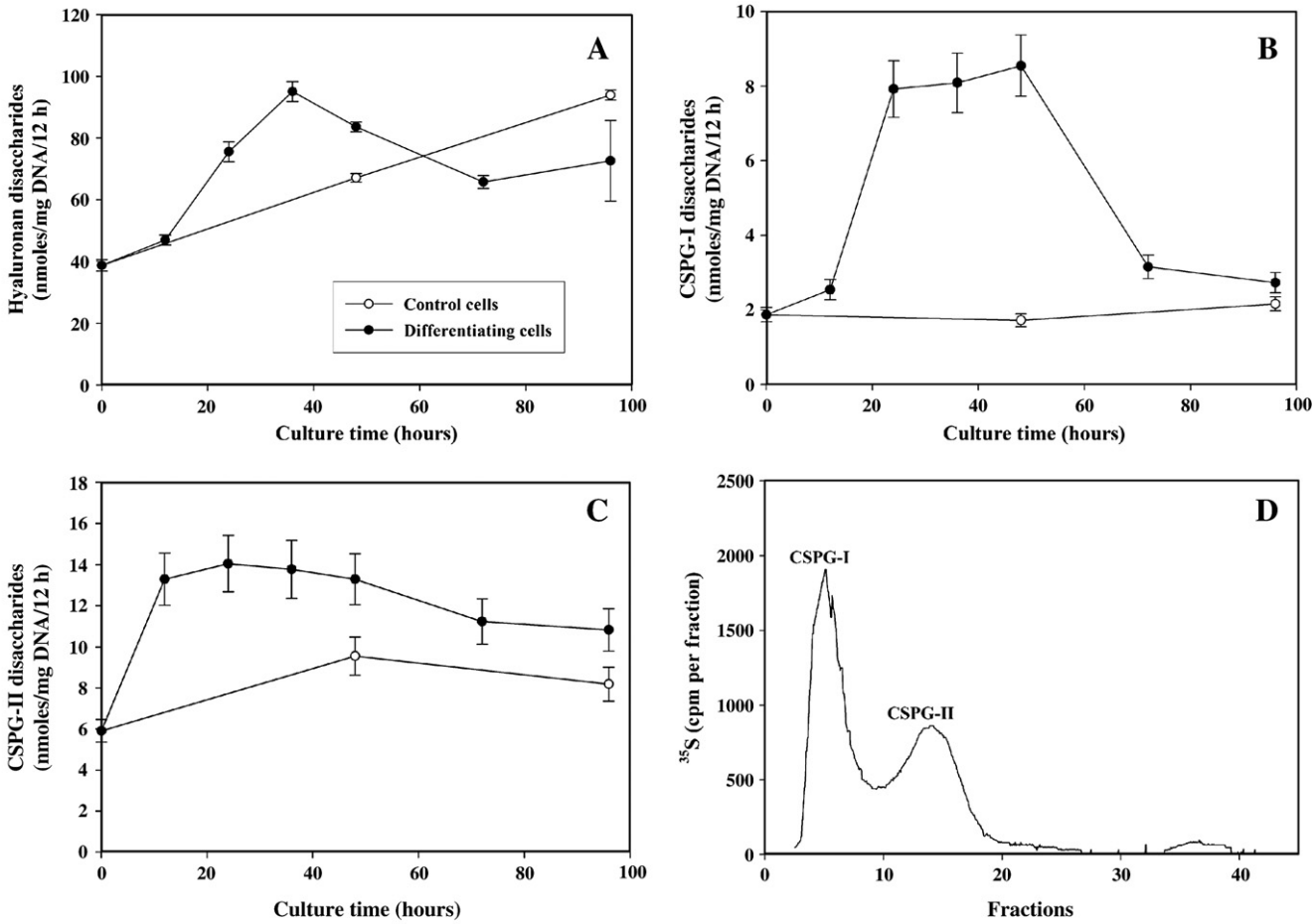

Fig. 3. Hyaluronan and chondroitin sulfate proteoglycan synthesis and release into the medium compartment at different times during differentiation. 3T3-L1 cells were metabolically labeled with [^{35}S]sulfate and [^{3}H]glucosamine and the medium was extracted for hyaluronan and proteoglycan purification. Chondroitin sulfate proteoglycans were further separated into a large and a small species by Superose 6 column. Open circles correspond to hyaluronan (Panel A) or proteoglycan (Panel B, CSPG-I or large proteoglycan and Panel C, CSPG-II or small proteoglycan) synthesized and released into the culture medium by control (no dexamethasone/IBMX treatment) cells. Closed circles correspond to cells cultured in the presence of the differentiation mixture for the times indicated in the figure and subsequent change to fresh culture medium containing insulin. A specific increase in the synthesis and release of CSPG-I during the differentiation (48 h in the presence of dexamethasone and IBMX) which decreases afterwards, can be clearly observed. On the other hand, CSPG-II appears to remain constant with little variation during the duration of the experiment. Panel D shows a representative elution profile of a Superose 6 column, separating both types of chondroitin sulfate proteoglycans (CSPG-I and CSPG-II).

insulin is added after 48 h of culture in the presence of IBMX and dexamethasone can be clearly observed. During this 48 h period, the cells start to differentiate and become preadipocytes (early marker-containing cells) (Rosen et al., 1978).

2.2. Hyaluronan and proteoglycan accumulation during 3T3-L1 differentiation

Fig. 2 shows the hyaluronan and chondroitin sulfate proteoglycan content in the medium at different stages of differentiation. During the 48 h of culture in the presence of IBMX and dexamethasone, the cells change their size and shape with shrinkage of the cytoplasm (Calvo et al., 1993) and no sign of triglyceride accumulation is observed (microscopically and biochemically). After the medium is changed and insulin added, the cells become round, and fat droplets appear during the next 48 h. Fat droplets increase in number and size until they coalesce, giving rise to mature adipocytes.

2.3. Time course of hyaluronan and chondroitin sulfate proteoglycan synthesis

Fig. 3 shows a detailed time curve of the synthesis of hyaluronan and chondroitin sulfate proteoglycans (CSPG-I and CSPG-II, according to Calvo et al. (1991)). Synthesis was maximal at 48 h of culture in the presence of IBMX and dexamethasone. After the medium was changed to fresh medium with insulin, there was a marked drop in the synthesis of the large proteoglycan fraction (panel B, CSPG-I) while hyaluronan (panel A) and the small proteoglycan (CSPG-II, panel C) remained almost constant. It is noteworthy that although hyaluronan synthesis in control cultures increased with culture time, maximum synthesis was reached later. This is in agreement with the fact that spontaneous differentiation of preadipocytes into adipocytes occurs more slowly than facilitated differentiation with IBMX and dexamethasone. Panel D in the same figure illustrates the elution profile on a

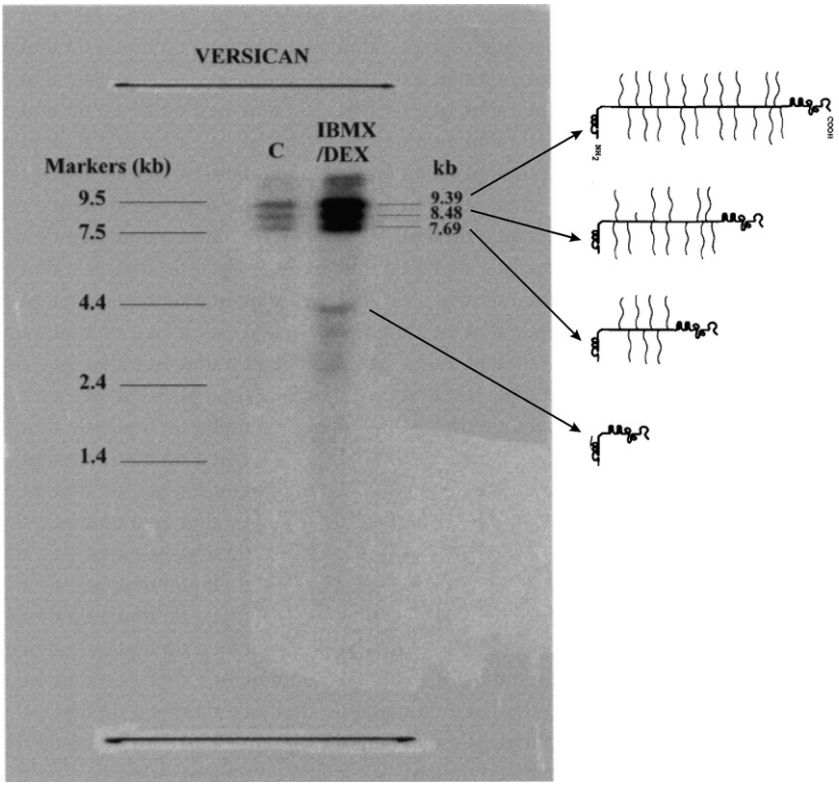

Fig. 4. Northern blot of a cDNA probe with the sequence for the human versican using a total RNA preparation from control and differentiated 3T3-L1 cells. Total RNA, prepared from 3T3-L1 cells as described in the methods section, was blotted against a radioactive labeled probe prepared with the cDNA sequence for human versican. An increased signal for the sample obtained from differentiated cells and the presence of three major bands, indicating a possible alternative splicing of the message, can be observed. To the right of the panel, a drawing of the corresponding putative proteoglycan species is presented.

Superose 6 column of the two chondroitin sulfate proteoglycans (CSPG-I and CSPG-II).

2.4. Time course of the appearance of chondroitin sulfate proteoglycan mRNA. Northern blot analysis

In a previous manuscript (Calvo et al., 1991), we characterized the large chondroitin sulfate proteoglycan (CSPG-I) as being probably related to the versican family. In order to investigate this possibility further, we used a cDNA constructed after the human sequence of versican, and corresponding to the 3′-region (about 1.3 kb, including untranslated region). When total RNA extracted from control cultures and cells that had been exposed to IBMX and dexamethasone for 48 h were analyzed by Northern blot procedure as described in Experimental Procedures, three bands were clearly identified (9.39, 8.48, and 7.69 kb, Fig. 4). The difference between control and treated cells showing the stimulation of RNA synthesis after treatment can be clearly observed. The right panel in the figure shows the possible structures derived from the alternative splicing, including a small (4.4 kb) species. In order to follow the time course of appearance of versican mRNA and correlate it with the metabolic labeling of "versican-like" proteoglycan, total RNA was extracted from cells at different stages of differentiation. Fig. 5 shows the increase in versican mRNA during culture in the presence of IBMX and dexamethasone and the disappearance of the signal after changing the culture

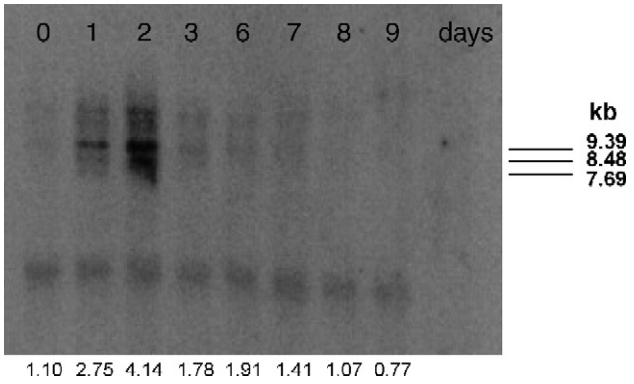

Fig. 5. Time course for the appearance of the mRNA for versican during differentiation of 3T3-L1 cells. Total RNA isolated from 3T3-L1 cells at different stages of differentiation was blotted against a human versican cDNA probe. This figure shows the increase and disappearance of the mRNA coincident with the metabolic labeling of the proteoglycan molecules, as shown in Fig. 3 (panel B) for CSPG-I. During days 1 and 2, the cells were cultured in the presence of dexamethasone and IBMX. For the remainder of the experiment, the cells were cultured with fresh medium containing insulin (10 μg/ml). The membrane was stained with ethidium bromide to look for differences in RNA load. Both the membrane and the autoradiography were photographed, scanned and digitized. As the different bands showed a similar behavior, all were combined for the purpose of image processing. The numbers below each lane represent the arbitrary values after normalizing the combined bands (9.39, 8.48 and 7.69 kb) for the total RNA load.

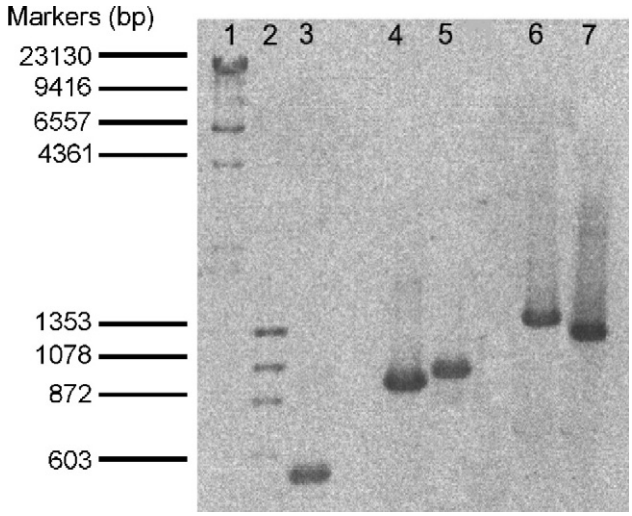

Fig. 6. RT-PCR analysis of RNA from differentiated 3T3-L1 cells. Total RNA isolated from differentiating (48 h culture in the presence of IBMX and DEX) 3T3-L1 cells was analyzed by RT-PCR using oligonucleotide primers synthesized according to the murine PG-M sequence. This figure shows the ethidium bromide negative photograph. Lanes 1 and 2: size markers (lane 1: λDNA-HIND III fragments and, lane 2: ∅X174RF DNA/HAE III fragments); lane 3: RT-PCR control; lane 4: amplification product corresponding to HA-binding zone, including regions A, B and B′ (spanning exons III, IV, V and VI) with a size of 926 base pairs; lane 5: α-region for chondroitin sulfate binding (exon VII), with a size of 1000 base pairs; lane 6: amplification product for lipoprotein lipase, as a control for differentiation; lane 7: β-region for chondroitin sulfate binding (exon VIII) with a size of 1250 base pairs.

medium. This correlates well with the metabolic labeling of "versican-like" proteoglycan; the larger size mRNA is the first to appear while the smaller species appears later. The numbers at the bottom of the lanes correspond to arbitrary units after digitizing the bands (considering all the spliced species together, due to the same behavior) and normalizing for the total RNA load.

2.5. RT-PCR analysis of hyaluronan binding and chondroitin sulfate attachment regions of 3T3-L1 proteoglycan

In order to further characterize the large proteoglycan molecule, we synthesized different sets of oligonucleotide primers designed after the mouse endothelial PG-M sequence (Yamagata et al., 1993; Ito et al., 1995) and amplified different portions of the molecule. Fig. 6 shows the RT-PCR products obtained using those primers. A hyaluronan binding sequence was observed, as well as the glycosaminoglycan attachment sites and the 3′ terminal region, containing EGF, lectin and complement-like portions. This was similar to the sequence recognized by the cDNA probe used in Figs. 4 and 5.

2.6. Sequence analysis of HA binding region of CSPG-I and chondroitin sulfate binding domain β

The sequences of the PCR products obtained using primers directed against the HA binding domain of murine PG-M and the chondroitin sulfate attachment domain β of the same proteoglycan were compared for DNA homology with the NCBI database, using BLAST routine. A 100% homology was observed for both of them with only the murine PG-M proteoglycan (accession number D28599); very little homology was observed with other sequences in the same database. The HA binding domain (Panel A) was 100% homologous to the region between bases 374 and 548 and for the chondroitin sulfate attachment region (Panel B) we were able to obtain a 94% homology with the region enclosed by bases 3857 and 3998 on the original PG-M sequence.

Panel A:
1 tggtctaaga-ggaagtggacaaaaatggaaaagatataaaggagacgactgtcttggtg 59
60 gcccagaacggaaatatcaagattggtcaggactacaaggggcgagtgtccgtgcctaca 118
119 catcccgatgatgtaggtgatgcttccctcaccatggtcaaactccgggctagtg 172
Panel B:
1 atgagcattctaacttcattggcacatcttccattctttcatggtcttccattttatcacctaagatgcttgttcctaag 80
84 ccatgtgagatgctcagcatacaatgtgctaatg 116
122 catgtcagtca 132

2.7. Analysis of HA association to CSPG-I

As we were able to amplify and detect the HA binding region in 3T3-L1 proteoglycan, we next performed an association experiment. In order to allow isolated radiolabeled CSPG-I to bind to HA we used aA1 (containing HA, HA binding region from aggrecan and link protein) as a carrier. Fig. 7 shows the results from a Sephacryl S-1000 analysis of the association product, compared to the product without aA1 as a control. It can be observed that 13% of the total radiolabeled proteoglycan binds to HA and moves towards the void volume of the column when in the presence of aA1.

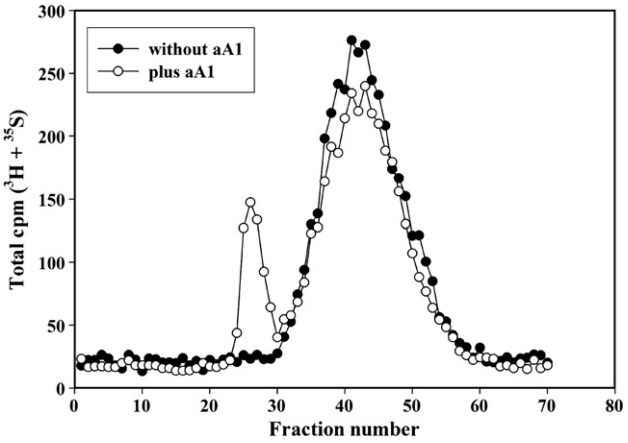

Fig. 7. Association of HA to aA1 and CSPG-I. After metabolic labeling of differentiating 3T3-L1 cells, the medium extract was chromatographed through Q-Sepharose and eluted with a linear 0.3–1.3 M NaCl gradient, as described. The proteoglycan peaks were collected, concentrated with Q-Sepharose, and eluted with 4 M guanidine HCl for further purification through a Superose 6 column. The peak corresponding to the large chondroitin sulfate proteoglycan (CSPG-I) was isolated, mixed with aA1 fraction (containing the proteoglycan aggrecan with its HA binding region, HA and link protein) or buffer, dialyzed against 0.5 M sodium acetate buffer, pH 7.1 and, then, chromatographed using Sephacryl S-1000, eluting with 0.5 M sodium acetate buffer, 0.5% Triton X-100, pH 7.0. Fractions were collected and radioactivity was measured with a liquid scintillation counter.

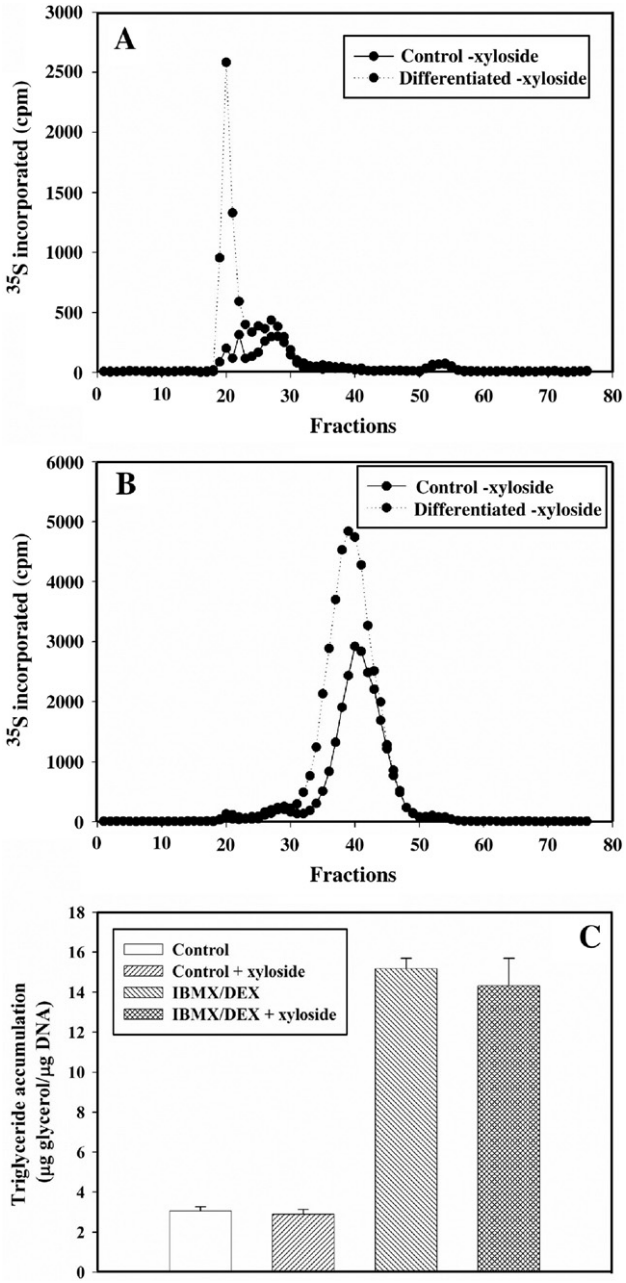

Fig. 8. GAG and triglyceride accumulation in the presence of xyloside. Panel (A) shows the elution profile of media extracts after confluent 3T3-L1 cells (both control or differentiated) were cultured in the presence or absence of 1 mM *p*-Nitro β-D-xylosyl pyranoside for two days prior to addition of dexamethasone and IBMX. With every medium change, xyloside was added until the desired degree of differentiation was attained. The profiles show the increase in free glycosaminoglycan chains for both control and differentiated cells, when xyloside was present. Panel (B) shows the triglyceride accumulation after the same treatment. Triglycerides were determined as described in the Experimental Procedures section.

2.8. Effect of exogenous xyloside on 3T3-L1 cell proliferation and differentiation

In order to investigate if some of the effects observed on 3T3-L1 cells were due to the complete versican molecule or to the glycosaminoglycan chains, a xylose derivative was used to further enhance GAG biosynthesis and reduce their insertion into the proteoglycan core. Fig. 8, panel A, shows the increase in free GAG chains and the concomitant decrease in proteoglycan-associated chains, after xyloside treatment. Fig. 8, panel B, shows the triglyceride accumulation observed in the presence of 1 mM *p*-Nitrophenyl β-D-xylosyl pyranoside. On a per DNA basis, no difference was observed in triglyceride accumulation when the proteoglycan structure was disrupted by the addition of the exogenous acceptor of the glycosaminoglycan structure. On the contrary, the continuous presence of 1 mM *p*-Nitrophenyl β-D-xylosyl pyranoside starting at preconfluent stages, resulted in a decreased proliferation rate (Fig. 9).

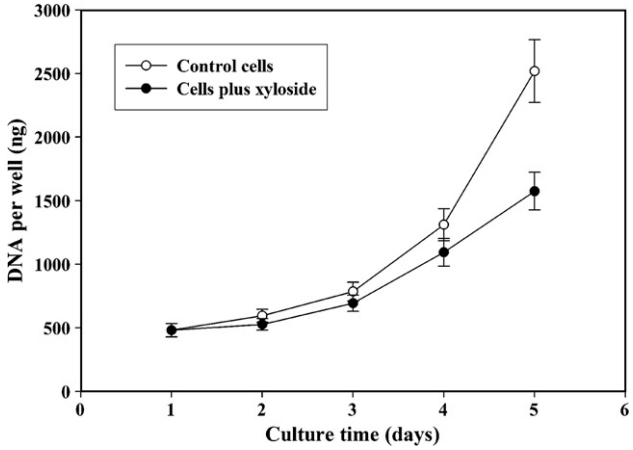

Fig. 9. 3T3-L1 cell proliferation in the presence of xyloside. Preconfluent cultures of 3T3-L1 cells were treated with 1 mM *p*-Nitro β-D-xylosyl pyranoside and DNA was determined on each well at different culture times. At day 0, 4000 cells were seeded in 24-well culture plates and xyloside treatment started after 24 h. With every medium change, the same amount of xyloside was added to the cultures. Cells in culture medium without xyloside were used as controls. All cultures received 10% FBS. At each culture time, DNA was determined in the individual wells, fluorometrically with the Hoeschst stain.

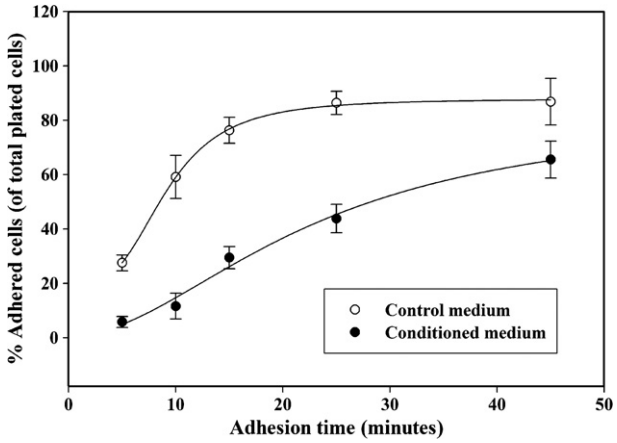

Fig. 10. 3T3-L1 cell adhesion on substrates from control or conditioned media. Substrates for cell adhesion were prepared from control or conditioned culture media on 24-well plates. After overnight exposure of plastic to the culture media, liquid was removed, plates were washed with phosphate-buffered saline solution and suspended cells (100,000 cells per well) were plated and DNA determined at different adhesion times.

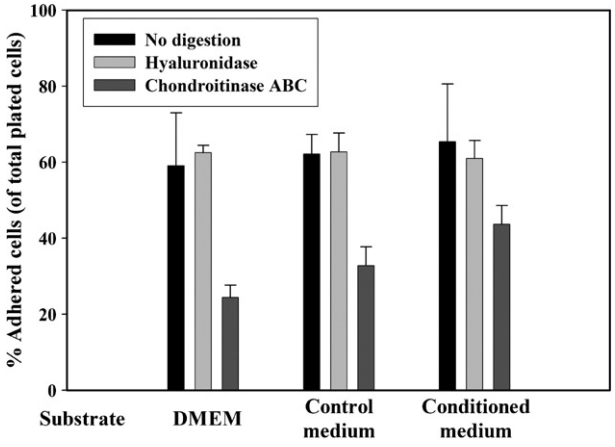

Fig. 11. Enzymatic treatments of substrates from control or conditioned media and the effect on cell adhesion. Control or conditioned media were treated with hyaluronidase or chondroitinase ABC, prior to their use for substrate coating of 24-well plastic plates, as indicated in Fig. 10. After coating, 3T3-L1 cells were added to each well (100,000 cell per well) and DNA was determined after 15 min of adhesion.

2.9. Effect of substrate on 3T3-L1 cell adhesion

Fig. 10 shows the different adhesion rates of 3T3-L1 cells to substrates obtained from control culture medium or conditioned medium of differentiating cells. Substrate from conditioned medium reduced cell adhesion for both control and differentiating 3T3-L1 cells in a similar fashion (data not shown). This suggests that the decrease in cell adhesion is rather related to the substrate than to changes in the cell membrane due to the differentiation process. When the media were digested with chondroitinase ABC, an enzyme that digests both hyaluronan and chondroitin sulfate chains, cell adhesion was partially restored (Fig. 11); if a specific hyaluronidase was used, this effect was less marked.

3. Discussion

3T3-L1 fibroblasts grow in culture until confluence is reached. At this point, proliferation is arrested and if culture continues beyond this point in the presence of 10% fetal bovine serum, markers of early adipocytic differentiation appear and the cells become preadipocytes. By a mechanism still not well understood, these preadipocytes escape arrested growth and proliferation resumes for at least one cycle. At this stage, late and very late markers of differentiation are induced and the cells become adipocytes, with triglyceride accumulation in fat droplets. As with other fibroblasts (Yoneda et al., 1988a,b) it seems that an increase in hyaluronic acid synthesis accompanies this resumption of proliferation by 3T3-L1.

We have demonstrated that hyaluronan synthesis correlates with triglyceride accumulation and appears at the stages where early adipocyte markers develop (Spooner et al., 1979). As we have previously shown (Calvo et al., 1993) the increase in hyaluronan synthesis is accompanied by a dramatic change in the viscoelastic properties of the culture medium, indicative of the formation of a complex network of macromolecules with the probable involvement of a link protein and/or a hyaluronan-binding proteoglycan. In the present manuscript, we show that a chondroitin sulfate proteoglycan mRNA can be detected using a probe directed against the sequence of human versican. This mRNA appears to be stimulated during culture in the presence of IBMX and dexamethasone (differentiation into preadipocytes) and follows an appearance and disappearance curve consistent with the metabolic labeling of the proteoglycan molecule. When we first described this proteoglycan (Calvo et al., 1991), we observed that after chondroitinase ABC treatment, three core protein bands could be clearly seen (370, 307 and 210 kDa), with a fourth one of less intensity. In this manuscript, we show that the message for the "versican-like" molecule appears split into three species. This is in agreement with a reported manuscript for proteoglycan PG-M (chick homologue of versican) from chick fibroblasts (Shinomura et al., 1995) where alternative splicing into four forms was observed and with other reports by the same group (Ito et al., 1995; Shinomura et al., 1995), indicating the presence of alternative splicing in PG-M like proteoglycan produced by mouse endothelial cells. They showed that multiple forms of the core protein could be generated from a primary PG-M transcript, and the sizes observed for the different RNAs, 10, 9 and 8 kb, are in perfect agreement with the sizes shown in this manuscript. In addition, they found larger sizes mRNAs, depending on the tissue analyzed, which also coincides with the faint bands observed in our study. Regarding the lower sized band and smear observed in the blot, this is consistent with the findings by Ito et al. (1995) where they indicated that bands with 4.7 kb or less could be due to further splicing to smaller forms or some artificial degradation of RNA. As indicated in the manuscript by Shinomura et al. (1995), the chondroitin sulfate attachment region is encoded by two exons (VII and VIII, spanning approximately 3 and 5 kb, respectively) and our transcripts would correspond, then, to PG-M (V0) when both exons are present, PG-M (V1) or PG-M (V2) when either one is absent. The smaller band could correspond to PG-M (V3) when both exons are spliced out. It is conceivable to think that each spliced form may have distinct functions and would be interesting to study their expression under different conditions.

The decrease in the rate of synthesis of CSPG-I ("versican-like" chondroitin sulfate proteoglycan) after withdrawal of the differentiation mixture is coincident with the observation by Musil et al. (1991) although they reported a larger change in the synthesis of the small proteoglycan (CSPG-II in our previous manuscript (Calvo et al., 1991)) compared to CSPG-I.

It is well known that some extracellular matrix proteins present a variety of forms after alternative splicing and that these forms could be developmentally regulated (Boyd et al., 1993). Different forms could play different roles during tissue organization as well as be involved in the regulation of cell–cell or cell-substrate adhesion.

The presence of an HA-binding region, determined by RT-PCR using specific primers and our previous results on the physicochemical characteristics of the large proteoglycan (Calvo et al., 1993), indicate a possible interaction between CSPG-I and hyaluronan, leading to the formation of a viscoelastic network during adipose differentiation. The good correlation between the

size of the fragments observed after RT-PCR and the expected results according to the sequence used for primer synthesis, indicated that the 3T3-L1 chondroitin sulfate proteoglycan could be the same as or closely related to the PG-M from endothelial murine cells and, thus, related to the versican family.

This possibility was further evaluated by sequencing two PCR products, corresponding to the HA binding domain and the chondroitin sulfate attachment domain β. The homology of both sequences with murine PG-M proteoglycan indicates that CSPG-I is, in fact, the same chondroitin sulfate proteoglycan. PG-M has been shown to be mainly expressed in developing tissues from embryonic origin as well as in tumor cells. The fact that the 3T3-L1 fibroblastic cells derive from mouse embryos correlates well with the presence of PG-M in these cells.

Although an HA binding region has been detected in PG-M, the binding of this proteoglycan to hyaluronan has not been tested directly. Binding properties have been inferred from the nucleotide sequence or indirectly through binding to fibronectin or inhibition of cell adhesion to different substrates (Yamagata et al., 1989; Yang et al., 1999). PG isolated from ovary was unable to aggregate to hyaluronan, possibly due to a weaker binding affinity compared to aggrecan. Our attempt to perform a direct binding experiment in this manuscript resulted in a 13% association. This low percentage could be a result of various factors, such as the existence of proteoglycan isoforms with different affinities for the glycosaminoglycan or the presence of detergent in the binding mixture during dialysis which could prevent a complete association. The direct binding of a proteoglycan to hyaluronan has only been achieved with good yield for aggrecan in the presence of link protein and thus better results should probably not be expected. On this account, previous results by Chang et al. (1983) when purifying proteoglycans synthesized by smooth muscle cells, show a similar reaggregation.

Having determined the molecular identity of CSPG-I as PG-M, its involvement in the differentiation process remained to be assessed. The properties of a proteoglycan molecule can be due to the intact molecule, or to either the core protein or the glycosaminoglycan chains. Incubation of cells with an exogenous xyloside acceptor, resulted in a disassembled proteoglycan molecule with the core protein separated from the carbohydrate chains. The results obtained in this manuscript indicated that the intact molecule was not necessary for triglyceride accumulation, when expressed on a DNA basis. However, results not shown, indicated that the DNA content in the incubation wells was lower in the presence of xyloside, observation which prompted us to perform a proliferation curve in its presence. The results obtained showed a marked reduction in proliferation when cells were cultured in the presence of this exogenous glycosaminoglycan acceptor, which could be due to a reduced adhesion of new cells to the plate surface. It has been reported that PG-M increases cell proliferation through protein domains G3 and G1 (Zhang et al., 1999). The G3 domain could act via EGF receptors, using EGFs characteristic signaling pathways and G1 would probably stimulate cell division as a consequence of the proteoglycan's ability to decrease cell adhesion. Similarly, in neural crest cells, versican regulates migration through its G3 domain (Perissinotto et al., 2000).

To evaluate cell adhesion, we coated plastic surfaces with culture medium obtained from cells undergoing chemical differentiation, where hyaluronan and chondroitin sulfate proteoglycan content is increased. The presence of probably both hyaluronan and proteoglycan in the culture plate resulted in a reduced cell adhesion. This could be due to an interaction of these molecules, individually or as a complex, with adhesion molecules at the cell membrane level. Which molecules are involved remains to be answered, and experiments to elucidate this matter are now in progress in our laboratory.

Although these results were observed for the "chemical" differentiation of 3T3-L1 preadipocytes, they suggest the possibility of a specific role for hyaluronan and chondroitin sulfate proteoglycan in the process of adipocyte differentiation. Even though this manuscript did not specifically address the relationship between glycosaminoglycan synthesis and adipocyte differentiation as a cause–effect phenomenon, it raises an interesting question as to the extent of this correlation. Experiments are now in progress to determine the role CSPGI/PG-M and hyaluronan in the formation of a fluid extracellular matrix network and its possible role in cell proliferation, migration and differentiation, in an attempt to assess at the molecular level the degree of interaction with membrane components and intracellular signaling.

Of special interest is the presence of the EGF-like domains in the proteoglycan and the possibility of alternative splicing, leading to proteoglycan forms that keep these domains but have lost the glycosaminoglycan-binding portion of the protein core, focusing in particular on the smaller spliced form (4.4 kb). As indicated for other cell types (NIH-3T3 and chondrocytes) (Yang et al., 1999; Zhang et al., 1999), these EGF-like domains could be responsible for 3T3-L1 proliferation prior to their differentiation. So far, to our knowledge, no EGF is produced by these preadipocytes and, thus, the protein core of this proteoglycan, through its EGF-like domains could be acting as growth factor. This possibility is currently under investigation in our laboratory.

4. Experimental procedures

4.1. Materials

Guanidine HCl and urea were purchased from Life Technologies/Bethesda Research Laboratories. [^{35}S]Sulfate (43 Ci/mg), D-[6-^{3}H]glucosamine (29.5 Ci/mmol) were from Du Pont—New England Nuclear. Sephadex G-50 (fine), Q-Sepharose, prepacked Superose 6, and Sephacryl S-1000 were from Pharmacia LKB Biotechnology Inc. Triton X-100 was from Pierce Chemical Co., and CHAPS[1] was from Calbiochem. Partisphere 5-PAC was from Whatman.

4.2. Cell culture

3T3-L1 cells (embryo, mouse, ATCC CCL 92.1) were purchased from the American Type Culture Collection. Cells were cultured at 37 °C under 5% CO_2, 95% air in 100-mm plastic culture dishes in Dulbecco's modified Eagle's medium

(GIBCO) containing 25 mM glucose and 10% fetal bovine serum (GIBCO, INOVAR). Cells were subcultured at 80% confluence as previously described (Spooner et al., 1979; Chernick et al., 1986; Calvo et al., 1991, 1993).

4.3. Induction of 3T3-L1 differentiation, metabolic labeling of cell cultures, and extraction of proteoglycans and hyaluronan

Cells were plated at a density of $1–2\times10^5$ cells/35-mm diameter dish. After reaching confluence, the medium was replaced with fresh medium containing 0.5 mM IBMX (Sigma Chemical Co.) and 0.1 μM dexamethasone (DEX, Sigma Chemical Co.) to induce differentiation (Smith et al., 1988; Calvo et al., 1991, 1993); control cells received fresh medium without any additions. At different times, cultures received 100 μl of a mixture of [^{35}S]sulfate and [^{3}H]glucosamine (50 μCi of each isotope/ml of final medium) in Dulbecco's modified Eagle's medium and culture continued for an additional 12 h. After 48 h in the presence of IBMX and dexamethasone, the medium was changed to fresh medium containing insulin (10 μg/ml of medium). The cells were labeled as indicated, for 12 h periods, up to 96 h. Medium was changed every 48 h. The medium was separated from the cells and protease inhibitors (10 mM *N*-ethylmaleimide and 1 mM phenylmethanesulfonyl fluoride) (Oike et al., 1980) as well as sufficient solid guanidine HCl (4 M final), were added to the medium.

Total ^{35}S incorporation into macromolecules was <0.2% of the total radioactivity added. More than 90% of the incorporated ^{35}S was recovered as chondroitin sulfate. Therefore, quantification of ^{35}S incorporation was expressed as equivalents of chondroitin sulfate synthesized. The calculation of ^{35}S-labeled chondroitin sulfate was based on total ^{35}S incorporation, the specific activity of [^{35}S]sulfate in the medium, and an average Mr of 500 for the repeating chondroitin sulfate disaccharide (Yanagishita et al., 1989a). This calculation is based on the assumption that the specific activity of [^{35}S]sulfate in the medium is not significantly diluted by endogenous sulfate sources (Imai et al., 1994). Correction for changes in specific activity of [^{3}H]glucosamine was performed using the ^{3}H/^{35}S ratios, according to Yanagishita et al. (1989b).

4.4. Quantification of hyaluronan and proteoglycans

Guanidine HCl extracts of medium samples were chromatographed on Sephadex G-50 (fine) columns equilibrated with 4 M guanidine HCl, 50 mM sodium acetate, pH 6.0, containing 0.5% (w/v) Triton X-100 to remove unincorporated isotopes (Yanagishita et al., 1987). An aliquot of the excluded macromolecular fraction was reapplied to another Sephadex G-50 (fine) column equilibrated with 0.1 M Tris acetate buffer, pH 7.3. The excluded fractions were digested with chondroitinase ABC (*Proteus vulgaris*, Seikagaku America) (0.1 unit/ml) in 0.1 M Tris acetate, pH 7.3 for 1 h at 37 °C. After incubation, the samples were divided into two aliquots: one was chromatographed on Sephadex G-50 (fine) equilibrated with 4 M guanidine HCl buffer to determine the fractions digested and not digested by the enzyme. The other fraction was centrifuged through Millipore filter with a cut off MW of 5000 in order to separate the chondroitin sulfate and HA disaccharides from the undigested material and the enzyme. For analysis of the disaccharide content in the chondroitinase ABC digest, samples were separated on an HPLC column (0.46×12.5 cm) of Partisphere 5-PAC (Whatman) (24,25). The effluent was monitored for UV absorbance at 232 nm to detect unlabeled disaccharides added as internal standards, and each 1-min fraction was counted for radioactivity. The column was calibrated with appropriate chondroitin sulfate disaccharide standards (Seikagaku America).

4.5. Northern blotting of the versican probe

Total RNA, from approximately 6×10^7 cells, was obtained according to the method described by Chomczynsky and Sacchi (1987). For the Northern blot analysis, 10 μg of the above RNA preparation were electrophoresed in a 1% agarose gel, containing MOPS and formaldehyde. The electrophoresis was performed at 50 V (constant) until the sample entered the gel, and 20 V overnight, until the dye reached 0.5–1 cm from the bottom of the gel. The gel was transferred to a 0.25 M ammonium acetate solution and gently shaken for 30 min, at room temperature. The gel was stained with ethidium bromide, and photographed under UV light. The RNA was transferred to a nylon membrane, eluting with 20× SSC solution. The RNA was fixed to the membrane with UV light.

The membrane was placed in a pre-hybridization buffer, containing: 50 ml of SSC solution (20×), 10 ml of Denhardt's solution, 10 ml of 10% SDS, 4 ml of sperm salmon DNA (10 mg/ml), 2 ml of 1 M Tris, 20 g of dextran sulfate and 124 ml of water to bring the volume to 200 ml. The pre-hybridization buffer was boiled for 3 min before use. The membrane was left in the buffer (approximately 50 ml) overnight, at 60 °C with constant shaking.

The cDNA probe (human versican, Telios Pharmaceutical Inc., San Diego, CA, 1.3 kb including nucleotides 1475–2802) was dissolved in Tris–EDTA buffer to a final concentration of 10 ng/μl). 2.5 μl of this solution were added to 20.5 μl of water and 10 μl of primer solution (random hexamers) were added. The solution was heated at 100 °C for 5 min, centrifuged at room temperature and 10 μl of 5× dCTP-buffer were added, along with 5 μl of [^{32}P]CTP and 1 μl of Klenow fragment. The reaction was allowed to proceed for 10 min at 37 °C. The labeled product was purified through a Sephadex G-50 column eluting with Tris–EDTA buffer.

After the pre-hybridization was complete, 50 ml of fresh hybridization buffer were added to the membrane plus the radioactively labeled probe. The hybridization was allowed to proceed overnight, at 60 °C with constant shaking. The membrane was washed with higher stringency up to 0.1×SSC, at 60 °C and analyzed by autoradiography.

4.6. RT-PCR analysis of the chondroitin sulfate proteoglycan mRNA

RT-PCR was performed using total RNA (1–5 μg) isolated from differentiated 3T3-L1 cells, using GIBCO BRL Super-

Script Preamplification System (Life Technologies, GIBCO BRL, Gaithersburg, MD) for the synthesis of the first cDNA strand, and oligo-dT as primers. PCR of this product was performed using the Elongase Amplification System (Life Technologies, GIBCO BRL, Gaithersburg, MD) using 10% of the RT (reverse transcriptase) final reaction and specific oligonucleotide primers. Primers were constructed according to the PG-M murine endothelial chondroitin sulfate proteoglycan sequence obtained from GeneBank and using the software Primer Detective (Clontech).

The primers used were: HA binding region (spanning exons III, IV, V, VI): Sense 5′-tctctgtctggaaaagtggtcc-3′, Antisense 5′-ggcatcaaatctgctatcagggg-3′, 926 bp; α region for chondroitin sulfate binding (spanning exon VII): Sense 5′-ctctacatcttcctcagagtcc-3′, Antisense 5′-agtatatgttgacccctccccc-3′, 1250 bp; β region for chondroitin sulfate attachment (spanning exon VIII): Sense 5′-tccaacagtgtcttatccaacg-3′, Antisense: 5′-agtccatgagcttcacgaaagg-3′, 1000 bp; lipoprotein lipase: Sense 5′-agtttggctccagagtttgacc-3′, Antisense 5′-ttcttccagagacttgtcatggc-3′). The PCR products were analyzed by agarose gel electrophoresis, ethidium bromide staining with UV detection, and Polaroid photography.

4.7. RT-PCR and sequence analysis of the DNA products

Two DNA fragments were obtained by RT-PCR (Preamplification System and Elongase Amplification System kit, Gibco Life Technologies) of total RNA from differentiating 3T3-L1 cells as described above. By using a previously published cDNA sequence (NCBI database accession number D28599), encoding for the core protein of murine PG-M, two sets of primers were obtained (Primer detective, Clontech). One set was used for partial amplification of a zone from exons III to VI, encoding for a link protein-like domain, leading to a 926 bp fragment (Primer A, sense: 5′-tctctgtctggaaaagtggtcc-3′; Primer B, antisense: 5′-ggcatcaaatctgctatcagggg-3′). The PCR conditions were 35 cycles at 94 °C for 30 s, 65 °C for 30 s and 68 °C for 1 min. The second set of primers was used for partial amplification of exon VIII, encoding for the chondroitin sulfate attachment domain β, with a product of 1250 bp (Primer C, sense: 5′-tccaacagtgtcttcacgaaagg-3′; Primer D, antisense: 5′-agtccatgagcttcacgaaagg-3′). PCR conditions were similar to the ones described for the other set of primers, except for the elongation period (68 °C for 2 min). Both PCR products were purified by electroelution after electrophoresis in 1% agarose gel and concentrated by precipitation with 0.1 volume of 3 M sodium acetate, pH 5.2 and 1 volume isopropanol. Then, fragments were purified with a mixture of phenol:chloroform: isoamyl alcohol (25:24:1), pH 7.9 and precipitated once. For sequencing of the PCR products, a commercial kit (SequiTherm Cycle Sequencing Kit, Epicentre Technologies) was used. Five hundred fmoles of the fragments, 25 pmoles of primer A or D, as needed, and 10 μCi of [^{35}S]-ATP were mixed with the provided buffer and dideoxynucleotides–nucleotides mix. After a 5 min period at 95 °C the mixture was amplified for 30 cycles at 95 °C for 30 s, 65 °C for 30 s and 70 °C for 1 min. The products were separated by electrophoresis in 6% polyacrylamide gel, containing 42 g urea/100 ml, and the sequence read from the autoradiography.

4.8. Association experiments

3T3-L1 cells were differentiated with IBMX and DEX, and metabolically labeled as described above. Medium extract was prepared and the proteoglycan fractions were obtained after elution from a Q-Sepharose column with a 0.3–1.3 M NaCl gradient, as previously described (Calvo et al., 1991). As the starting NaCl concentration was 0.3 M, the hyaluronan eluted along with the unbound material. Fractions containing the ^{35}S-labeled proteoglycans were collected and diluted with an equal volume of 8 M urea. One ml of Q-Sepharose gel was added to each sample, left overnight at 4 °C and the proteoglycan eluted and concentrated with 4 M guanidine HCl. Five hundred microliters of medium extract were applied to a Superose 6 column and eluted with 4 M guanidine HCl, 0.5% CHAPS, 50 mM sodium acetate buffer, pH 7.0. The peak corresponding to CSPG-I was collected and concentrated to 1 ml. Two 500 μl aliquots were separated and one received 75 μl of a 10 mg aA1/ml solution, in 4 M guanidine HCl. aA1 fraction was a kind gift of Dr. Lawrence C. Rosenberg. These aliquots were dialyzed against 300 ml of 0.5 M sodium acetate, pH 7.1, overnight, with two changes of buffer. Five hundred microliters of each sample were chromatographed using a Sephacryl S-1000 column, eluting with 0.5 M sodium acetate, 0.5% Triton X-100, pH 7.0 and 0.4 ml fractions were collected. Radioactivity was determined by liquid scintillation counting.

4.9. Cell proliferation and differentiation in the presence of xyloside

p-Nitrophenyl β-D-xylosyl pyranoside (Sigma Chemical Co.) (1 mM, final concentration), was added to confluent 3T3-L1 cultures, two days prior to inducing the differentiation process with IBMX and dexamethasone. Each time the medium was changed, xyloside was added. Differentiation was assessed by triglyceride accumulation. Preconfluent cells (4000 per well in 24-well plates) received 1 mM *p*-Nitrophenyl β-D-xylosyl pyranoside the day after plating. DNA was determined on each well, after two washes with PBS, at different proliferation times, by direct addition of Hoeschst H33258 (0.1 μg/ml) in 10 mM Tris–HCl, 0.2 M NaCl, 100 μM EDTA, pH 7.4 at room temperature, for 10 min. DNA was determined spectrofluorometrically. Control as well as treated cells were cultured in the presence of 10% FBS.

4.10. Cell adhesion

Plastic culture plates (24-well plates) were coated with 300 μl of either culture medium or conditioned medium from cells cultured for 48 h in the presence of IBMX and dexamethasone. After overnight stand at room temperature, the medium was aspirated. Where indicated, the media were digested with 0.1 IU of chondroitinase ABC, or I IU of hyaluronidase from *Streptomyces hyaluronlyticus* (Calbiochem), for 1 h at 37 °C,

before the coating procedure. 3T3-L1 cells (100,000 per well) were added and incubated at different times, at 37 °C, in 5% CO_2. DNA was determined on each well, after two washes with PBS, as described previously using the Hoeschst technique. All media contained 10% FBS.

4.11. Triglyceride and DNA determination

The triglyceride content of the cells was used as the principal end point of preadipocyte differentiation. Cells were washed once with 0.15 M NaCl and 0.01 M phosphate-buffered saline at room temperature, and 1 ml of water was then added to each culture. The cells were scraped using a rubber policeman and transferred to a 15-ml plastic centrifuge tube. The cell suspension was sonicated at maximal output for 15 s with a micro tip, and 100 µl were saved for DNA determination. Triglycerides were determined using the Sigma colorimetric determination kit (Cat. No. 240; Sigma Chemical Co., St. Louis, MO) as instructed by the manufacturer.

DNA was determined in the aqueous sample (100 µl) using the diamino benzoic acid technique (Kissane and Robins, 1958; Hinegardner, 1971) or according to Downs and Wifinger (1983) using the Hoeschst reagent and salmon sperm DNA as standard.

4.12. Statistical analysis

All experiments were performed at least three times, each determination done in triplicates. Mean values with their standard errors were analyzed by ANOVA followed by Tukey–Kramer test for multiple comparisons. Differences were considered significant when $p<0.05$.

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