Galectin-3 gene (*LGALS3*) expression in experimental atherosclerosis and cultured smooth muscle cells

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Abstract The galectin-3 gene (*LGALS3*) encodes a β-galactose binding lectin. *LGALS3* expression is associated with neoplastic transformation and with differentiation of monocytes to macrophages. Factors involved in migration, proliferation, adhesion and differentiation of vascular smooth muscle cells (SMC) play a major role during atherosclerosis development. Expression of the galectin-3 gene was not detected in quiescent SMC but was activated in aortas of hypercholesterolemic rabbits, in aortas of rats after balloon injury and in cultured SMC. These results suggest that galectin-3 production is involved in the developmental process of atherogenesis.

Key words: Soluble galactose binding lectin; Experimental atherosclerosis; Reverse transcription-polymerase chain reaction; Promoter; Transfection; DNA methylation

1. Introduction

Galectin-3, a soluble β-galactoside binding lectin [1], is involved in various biological functions related to cell adhesion, proliferation and differentiation [2]. Galectin-3 is a non-integrin laminin binding protein [3] and plays a role in cell-cell or cell-matrix interactions [2]. The production of the lectin is also related to the proliferative state of different types of cells [4,5]. It is expressed in a large range of neoplasms and correlates with the metastatic potential of different tumors [6–8]. In the nucleus, it is found associated with hnRNP complexes [9,10] and has been shown to be necessary for RNA splicing [11].

These observations incited us to investigate the expression of the *LGALS3* (galectin-3) gene in smooth muscle tissues. Intimal proliferation of vascular smooth muscle cells (SMC) in the arterial wall is thought to play important roles during intimal formation and throughout the development of atherosclerotic plaques [12,13]. Accumulation of factors resulting in alteration of proliferation, migration, adhesion and differentiation of SMC may be critical for the evolution of the lesions. Expression of *LGALS3* has been analyzed in vivo in two experimental models of accelerated atherosclerosis and in vitro in cultured smooth muscle cells. We present evidence that *LGALS3* is not expressed in normal rabbit and rat arteries. However, expression of the gene is activated in aorta of hypercholesterolemic rabbits and in aorta of rats upon endothelial denudation by balloon catheter injury. Transcription is also very active in primary cultures of rabbit SMC as well as in spontaneously immortalized SMC lines. However, in some SMC lines transformed by herpetic viruses CMV and HSV-2 expression of *LGALS3* appears to be turned off. The mechanism involved in the control of gene expression is independent of DNA methylation.

2. Materials and methods

2.1. Animals

Hypercholesterolemia was induced by feeding male New Zealand White rabbits on a cholesterol-rich diet for a period of 6 weeks. The intima media was separated from the adventitia and endothelium before RNA extraction. Thoracic aorta of male Wistar rats was mechanically injured by three passages of an inflated embolectomy catheter (Fogarty, size 2F) introduced by the left common carotid artery under general anesthesia [14]. The animals were killed at varying times after injury, the aorta was opened longitudinally and the intima media was dissected free from the adventitia using two pairs of fine forceps. Samples were removed from several rats and pooled at different time points after surgery.

2.2. Cell cultures

Primary cell cultures of SMC from aortas of New Zealand White rabbits were prepared as described [15]. The Rb-1 line is a SMC continuous line originating from the aorta of New Zealand rabbits [16]. Transformed SMC were obtained upon transfection of primary SMC cultures with plasmids containing the transformed region of SV40 [15], the morphological transforming region II of herpes simplex type 2 (HSV-2), [17] or the morphological transforming region of human cytomegalovirus (CMV) [18].

2.3. cDNA synthesis and PCR (RTPCR)

Total cell RNA (1 μg), isolated by the guanidinium isothiocyanate method [19], was reverse transcribed in a reaction mixture containing 8 units of AMV reverse transcriptase (Promega, Madison, WI) and 10 pmol of nonamer random primer. One tenth of the reactions was used as template for 30 PCR cycles using primers specific for the rabbit and rat *LGALS3* gene, the actin gene or the glyceraldehyde phosphate dehydrogenase (*GAPDH*) gene. Primer sequences were: rabbit *LGALS3*, upper primer = 5′ GTT ATC TGG GTC TGG AAA CC 3′, lower primer = 5′ TCT GTT TGC ATT GGG CTT CAC C 3′; rat *LGALS3*, upper primer = 5′GTC ATT GTG TGT AAC ACG AAG C 3′, lower primer = 5′ AGG GTT ATG TCA CCA CTG ATC C 3′; actin, upper primer = 5′ GCC GGG ACC TGA CCG ACT AC 3′, lower primer = 5′ AGG AAG GAG GGC TGG AAG AG 3′; *GAPDH* upper primer = 5′ GGC ACC GTC AAG GCT GAG AA 3′, lower primer = 5′ CCA CCA CCC TGT TGC TGT AG 3′. For quantitative procedures, PCR products were transferred to nylon membranes and hybridized to ^{32}P-labelled internal oligonucleotides specific for rat *LGALS3* or *GAPDH* cDNA. Dried gels were scanned using Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Quantification of *LGALS3* transcripts was evaluated with reference to *GAPDH* gene whose expression was constant throughout the experiment. Each sample was assayed in triplicate.

2.4. Southern and Northern blot analysis

DNA and RNA extractions, gel electrophoreses, and transfer to nylon membranes were performed according to standard techniques

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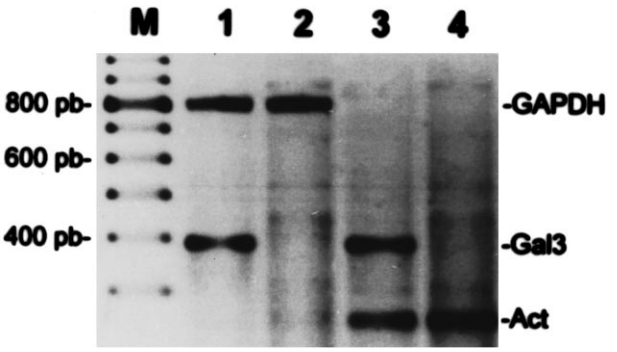

Fig. 1. Detection of *LGALS3* transcripts in aortas from normal and hypercholesterolemic rabbits. RNA from media of aortas from rabbits on a normal diet (lanes 2 and 4) or a cholesterol-rich diet (lanes 1 and 3) were analyzed by RT-PCR for the presence of *LGALS3* transcripts. PCR fragments were loaded on 1.5% agarose gels. *LGALS3* transcripts were coamplified with *GAPDH* (lanes 1 and 2) or with actin (lanes 3 and 4). The expected sizes for *LGALS3*, actin, GAPDH fragments are 396 bp, 256 bp and 800 bp respectively. Lane M is a molecular weight marker.

[20]. 20 μg of digested cellular DNA and 20 μg glyoxylated RNA were analyzed. Hybridization was performed as described [20] using *LGALS3* cDNA or genomic DNA probes. DNA methylation was analyzed using *Msp*I, a restriction enzyme sensitive to methylation of the outside cytosine in the sequence CCGG and *Hpa*II sensitive to methylation of the internal cytosine in the same sequence.

2.5. Transfections and luciferase assays

The plasmid pPG3.10 contains about 10 kb of the 5′-flanking region of rabbit *LGALS3* fused to the luciferase gene coding sequence [21]. pSV2-Luc, a plasmid in which the luciferase gene is driven by the SV40 early promoter [22], was used as a positive control for luciferase expression. Plasmid pSV-βGal (Promega, Madison, WI, USA) contained the β-galactosidase gene under the control of SV40 promoter and enhancer elements. This plasmid was included in each transfection assay and used as an internal standard of efficiency and reproducibility. Plasmids were transfected according to Midoux et al. [23]. Briefly, 10^6 cells were incubated for 4 h in DMEM containing 100 μM chloroquine, 2 μg of pSV-βGal and 5 μg of pPG3.10 or pSV2-Luc plasmid DNA complexed with 12 μg lactosylated poly-L-lysine. Cells were maintained in fresh DMEM supplemented with 10% SVF for 48 h after transfection. Luciferase was assayed using an automated luminometer (Berthold Lumat LB 9501, Colombes, France). β-Galactosidase was assayed as previously described [21].

3. Results

3.1. Detection of LGALS3 transcripts in aortas from normal or hypercholesterolemic rabbits

Media from aortas of both normal rabbits and cholesterol fed rabbits were analyzed for the presence of *LGALS3* transcripts. Because of the low amounts of RNA available, RT-PCR was used. In normal aortas, no signal was detected when amplification was conducted with *LGALS3* primers. Conversely, specific transcripts were detected when actin and GAPDH primers were used (Fig. 1). In aortas from hypercholesterolemic rabbits a signal specific for *LGALS3* transcripts was detected. Although no quantification can be done on this type of experiment, it appeared clearly that *LGALS3* was not expressed in the media from normal rabbit aortas but was induced in the intima media of hypercholesterolemic rabbit aortas. The *LGALS3* transcripts detected in aorta from hypercholesterolemic rabbits could be the result of expression in macrophages and/or SMC which are the main types of cells in these lesions.

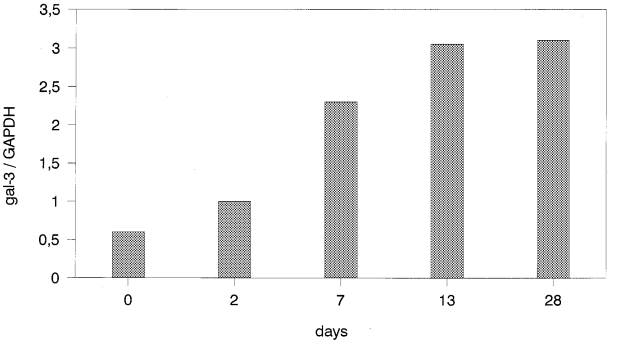

Fig. 2. Expression of *LGLAS3* after angioplasty of rat aortas. Thoracic aortas were subjected to injury with a balloon catheter. Intima media was analyzed at different time points after injury (*x*-axis; 0 stands for uninjured control aorta). *LGALS3* transcripts were analyzed by quantitative RT-PCR. Amount of transcripts was standardized against the amount of *GAPDH* transcripts.

3.2. Expression of LGALS3 after thoracic aorta angioplasty

Balloon catheter injury of rat thoracic aorta induces activation, migration and proliferation of SMC leading to fibromuscular thickening of the intima [13,14]. The aortas were analyzed at different times after surgery and transcription of *LGALS3* was followed by RT-PCR (Fig. 2). Only a minimal background level of *LGALS3* mRNA could be detected in non-injured aortas confirming the previous observations on control rabbit aortas. However, following injury, *LGALS3* transcripts were clearly present. Transcription reached a plateau 14–28 days after injury corresponding to maximal thickening of the intima, suggesting that activated SMC can induce transcription of *LGALS3*.

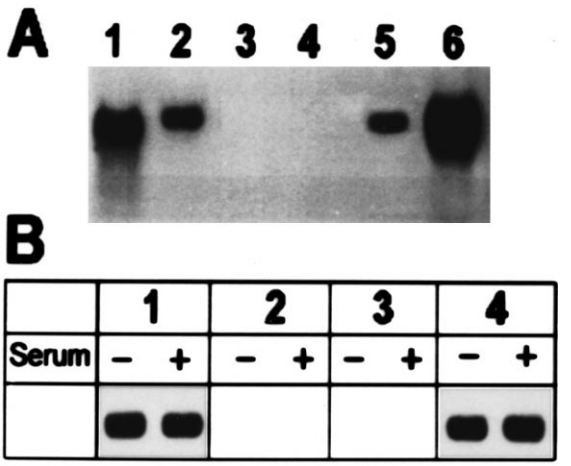

Fig. 3. Northern blot analysis of *LGALS3* transcripts in cultured rabbit smooth muscle cells. A: Northern blots of total RNA were probed with a *LGALS3* cDNA. Lane 1: cultures of primary SMC; lane 2: Rb-1, a spontaneously immortalized SMC line; lane 3: RCMV-5, a continuous SMC lines obtained upon transfection of transforming region I of CMV; lane 4: RBC-16, a continuous SMC lines obtained upon transfection of transforming region II of HSV-2; lanes 5 and 6: LSV3-4, LSV3-9, SV40 transformed cell lines. B: Influence of serum concentration on *LGALS3* expression. Rb-1 (lane 1), RCMV-5 (lane 2), RBC-16 (lane 3), and LSV3-4 (lane 4) were cultivated in medium containing 0.2% serum (lanes −) or 10% serum (lanes +).

3.3. Detection of LGALS3 transcripts in rabbit cultured smooth muscle cells

Northern blot analysis was performed on RNA isolated from different sources of SMC and probed with a *LGALS3* cDNA (Fig. 3A). Primary cultures of SMC from rabbit aortas, harvested after two passages, as well as the spontaneously immortalized rabbit SMC line Rb-1 [16] were found to transcribe the gene actively. All SV40 transformed SMC were also positive although expressing variable amounts of *LGALS3* transcripts. In most of the cell lines transformed by the morphological transforming region of herpetic viruses (CMV and HSV-2), the amount of *LGALS3* mRNA was comparable to that detected in Rb-1 cells (data not shown). However, in two of these lines, RCMV-5 and RBC-16, *LGALS3* mRNA was not detected (Fig. 3A) even using RT-PCR (data not shown). It appeared to be a stable trait and was not modified after repeated passages. Equal loading of total RNA on gels for Northern blot analysis was checked by probing the filters for β and γ actin transcripts (data not shown).

It has been reported that, in fibroblasts, the level of expression of galectin-3 was dependent on the proliferative state of the cells [24]; in quiescent cells galectin-3 was at a low level while upon stimulation by adding serum in the culture medium, cells expressed large amounts of galectin-3. In the case of SMC, no significant differences in *LGALS3* transcripts appeared when adding 0.2% or 10% serum to the culture medium (Fig. 3B). These results indicate that while *LGALS3* was not expressed in quiescent SMC of the media of normal arteries, it could be activated in cultured SMC. This expression appeared to be constitutive and independent of serum factors.

3.4. Transfection of LGALS3 in expressing and non-expressing cell lines

Differential expression of *LGALS3* in SMC may be surprising considering that the cell lines have the same lineage. No difference could be evidenced in the gene structure in Southern blot analysis (data not shown). Both expressing (Rb-1) and non-expressing (RCMV-5) lines are able to activate a reporter gene under the control of the regulatory region of *LGALS3* in transfection experiments (Fig. 4). This result indicates that transcription factors are present in both cell types and that sequences responsible for the differential expression are not present or not functional in the genomic region −10 kb/+112 bp. The possibility of a repressor acting on a genomic region not present in the reporter plasmid cannot be excluded. Alternatively, DNA methylation could be involved in this differential regulation. Methylation was analyzed, by Southern blotting, on *Msp*I/*Hpa*II sites upstream of the transcription initiation sites in Rb-1 and RCMV-5 DNA (Fig. 5). A difference in sensitivity of hybridization was detected even when using *Pvu*II, an enzyme not sensitive to site methylation. However, no difference could be detected in the banding pattern of these expressing and non-expressing cell lines. For both lines, the restriction sites immediately upstream of the transcription sites in the GC rich region of the promoter were unmethylated while sites located more upstream of this region were methylated. This indicates that methylation pattern of DNA was identical in the regulatory region of *LGALS3* in these cell lines.

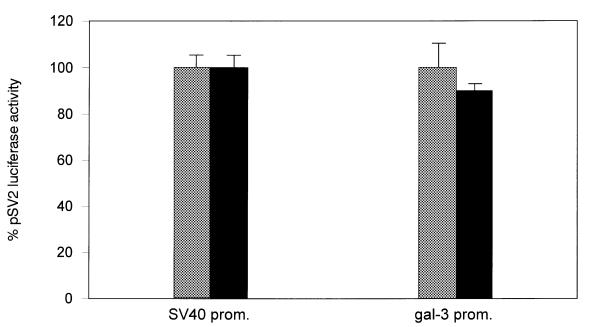

Fig. 4. Transfection of *LGALS3* cDNA in galectin-3 expressing and non-expressing SMC. pPG3.10, a plasmid containing about 10 kb of the 5′ flanking region of rabbit *LGALS3* upstream of the luciferase gene (gal-3 prom.), was transfected into Rb-1 (black bars) and RCMV-5 cells (gray bars). The luciferase activity was expressed as the percentage of that obtained in the reference experiment using pSV2Luc (SV40 prom.), a plasmid containing the luciferase gene under the control of the SV40 late promoter.

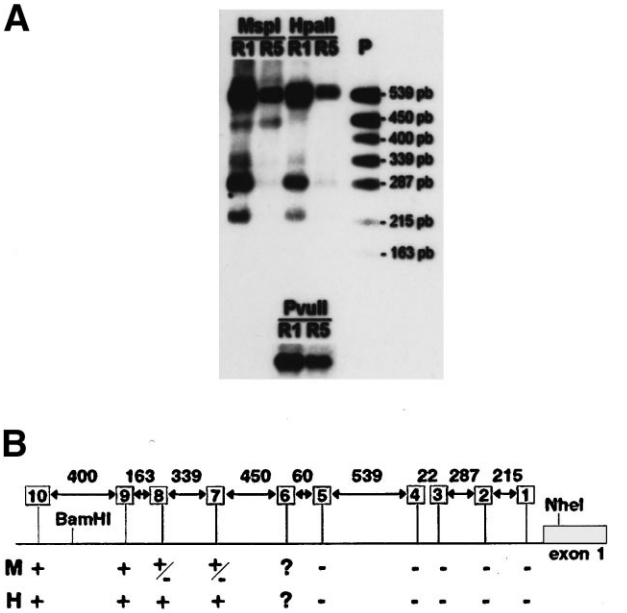

Fig. 5. Methylation pattern of *Hpa*II-*Msp*I sites of rabbit *LGALS3* in expressing and nonexpressing cell lines. A, upper part: Total cellular DNAs from Rb-1 (lanes R1) and RCMV-5 (lanes R5) were digested with *Msp*I or *Hpa*II and hybridized to the cloned *Nhe*I/*Bam*HI fragment containing about 2.4 kb of the 5′ flanking region of *LGALS3*. Lane P is the pattern obtained after cleavage of the *Nhe*I/*Bam*HI fragment with *Msp*I. Lower part: Southern blotting of the same DNA digested with *Pvu*II. B: Distribution of the *Msp*I/*Hpa*II sites along the 5′ flanking region of rabbit *LGALS3*. The sites are numbered from right to left starting from the site closest to exon 1. + and − respresent methylated and unmethylated sites respectively. M and H stand for *Msp*I and *Hpa*II sites, respectively. The sizes of the restriction fragments are indicated in bp.

4. Discussion

A hallmark of atherosclerosis is proliferation and migration of smooth muscle cells in the arterial intima. While expression of *LGALS3* is frequently enhanced in lesions involving cell proliferation, no studies have been conducted, to our knowledge, to investigate its expression in SMC of the normal and pathological arterial wall. No specific transcripts for *LGALS3* could be detected in media of aortas from normocholesterolemic rabbits but were easily evidenced by RT-PCR analysis in lesions of aortas from hypercholesterolemic rabbits. These

lesions are rich in foam cells which derive partly from monocytes/macrophages and partly from SMC [25]. Differentiation of monocytes to macrophages increases galectin-3 production [26,27] by means of gene activation [28]. Consequently, *LGALS3* transcripts could be the result of expression in macrophages and/or SMC.

Balloon catheter injury of artery is a model of accelerated arteriosclerosis, which is more related to restenosis after angioplasty than to atherosclerosis. However, this experimental model is a well defined system of SMC activation without any defined participation of monocytes/macrophages. Rat aortic injury induces a first step of medial SMC proliferation. DNA synthesis is maximal 2 days after treatment [13,14]. SMC migrate into the intima where their further proliferation and the production of extracellular matrix components result in definite thickening of the intima. *LGALS3* mRNA increased with time and reached a plateau at late time points when the intimal formation reaches a maximum [14]. These results indicate that activated SMC can produce *LGALS3* transcripts. It should be noted that a low level of *LGALS3* expression is detected 2 days after angioplasty when medial SMC are in active replication. At this early time point, the number of activated cells is low compared to the total amount of cells in the samples thus minimizing the efficiency of detection.

Expression of *LGALS3* transcripts is not detected in quiescent SMC from the media of normal vessels but raises a high level in primary cultures. This activation is probably related to a change in phenotype. SMC are in a contractile phenotype in the normal vessel and acquire a synthetic or secretory phenotype in cultures or in atherosclerotic lesions. This is associated with induction of a specific set of genes [13]. Our results indicate that *LGALS3* belongs to this class of genes activated during phenotypic modulation of SMC from the contractile to the secretory state. Once activated, *LGALS3* expression in SMC appeared to be constitutive and independent of serum concentration, a situation different from quiescent 3T3 cells in which addition of serum factors increased the expression of *LGALS3* both at protein and mRNA levels [29]. This suggests that regulation of *LGALS3* may be dependent on the cell type.

Most SMC established cell lines also expressed galectin-3. It has been reported that SV40 transformed fibroblasts expressed more lectin than their non-transformed counterparts [30]. In SV40 transformed SMC lines, the level of *LGALS3* transcripts is variable and not related to the rate of cell growth (data not shown). In some cell lines transformed by the morphological transforming region of human cytomegalovirus or HSV-2, expression of *LGALS3* was completely turned off. This appeared to be a stable character. The reason for decreased expression of *LGALS3* in these cells is not clear. The cloned promoter was equally active in expressing and non-expressing cells upon transfection of a reporter plasmid containing 10 kb of DNA upstream of *LGALS3*. This rules out the possibility that non-expressing cells lacked specific transcription factors. Also, no difference was observed in the methylation pattern of the GC rich region upstream of the gene. Alternatively, non-expressing cells could produce a negative transcription factor. The binding element for such a factor should be looked for in a region distant from the gene. Another explanation could be that SMC expressing different phenotypes are present in the rabbit normal artery from where these cell lines are derived. In the rat arterial wall, two different phenotypes, designated π and μ, have been demonstrated [13]. These phenotypes are stable and are maintained in cultures upon successive passages. The isolation of clones of transformed SMC expressing or non-expressing constitutively *LGALS3* could be related to the existence of such heterogeneity in the rabbit vessel wall.

The noticeable activation of *LGALS3* gene in cells involved in the development of atherosclerosis establishes a strong relationship between the expression of this factor and the development of plaques. While its precise function in atherosclerosis remains to be investigated, several lines of evidence suggest that this lectin could play a role in control of cell adhesion. Galectin-3 is considered a marker of macrophage differentiation. It is the major non-integrin laminin binding protein of macrophages [3] and could play a role in extravasation and tissue fixation of macrophages by modifying cell-cell and cell-matrix interactions [2]. Our results suggest that activated SMC may acquire specific traits of macrophages. This may not be surprising considering that SMC are known to express the macrophage scavenger receptor which mediates uptake of modified low density lipoproteins, a process thought to lead to the transformation of SMC into foam cell formation [31]. More recently, the expression of another marker has been described, the macrophage CD68 antigen in subendothelial SMC of both normal and atheromatous human aortas [32]. Galectin-3 expression in SMC supports these observations. Production of galectin-3 in SMC could act as an anti-adhesive factor in reducing the interactions between integrins and matrix glycoproteins thus facilitating their migration and proliferation in the neointima.

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