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Expression levels and functional aspects of the hyaluronan receptor CD44 Effects of insulin, glucose, IGF-I, or growth hormone on human arterial smooth muscle cells

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Abstract

An increased amount of hyaluronan (HA) in the arterial wall is a feature of the diabetic macroangiopathy. The functional consequences of accumulated HA are mediated through binding to CD44. The regulation of this receptor by diabetic metabolic and hormonal factors is, however unknown. The objective of this study was to examine the influence of glucose, insulin, insulin-like growth factor I (IGF-I), and human growth hormone (hGH) on the formation and function of the HA receptor CD44 in cultures of human aortic smooth muscle cells (SMCs). Migration of nonproliferating SMCs were determined by estimating the area covered by cells 6 days after removal of a barrier. Cellular content of standard CD44 and its isoforms, CD44v3 and CD44v6, and HA-binding capacity were measured using a modified enzyme-linked immunosorbent assay procedure. The analysis is made either with antibodies against CD44 or with HA as a ligand. The migration assay showed that glucose, insulin, and IGF-I were able to stimulate SMC migration ($2P < .01$). Anti-CD44 antibody inhibited the stimulated migration at most concentrations. Insulin increased HA binding at 100 to 1000 μU/mL insulin ($2P < .03$). CD44 expression was only elevated at 1000 μU/mL insulin ($2P < .03$), whereas CD44 content decreased at 2 ng/mL hGH and increased at 16 ng/mL hGH ($2P < .01$). Glucose and IGF-I reduced the amount of the variant isoform CD44v3 ($2P < .01$) but did not change the amount of total CD44. CD44v6 was not present on human arterial SMCs. In conclusion, the present data obtained with human arterial SMCs in vitro support a role of CD44 and its isoform, CD44v3, in the SMC response to the metabolic and hormonal disorders of diabetes.

1. Introduction

It is well established that there is a markedly higher frequency of cardiovascular disease among individuals with diabetes mellitus; diabetic women, in particular, are more predisposed to cardiovascular diseases [1,2]. It has not been possible to explain these observations by referring to classical risk factors as, for example, hypertension and hyperlipidemia [3,4]. As an alternative concept, the presence of a diabetic macroangiopathy has been suggested as a forerunner for the development of atherosclerosis. The diabetic macroangiopathy is considered to represent vessel wall changes, which are regarded to be a consequence of the diabetic metabolism. A number of morphological and biochemical studies have shown the presence of several abnormalities in the tunica media. Changes such as calcium deposition [5] and accumulation of periodic acid-Schiff–positive material, connective tissue [6,7], fibronectin [8], type IV collagen [9], and hyaluronan (HA) [10] are observed in the tunica media from patients with diabetes. These changes seem to be independent of and different from the alterations seen in presence of atherosclerotic plaque [11,12]. However, the pathogenesis of the diabetic macroangiopathy remains poorly understood.

Hyaluronan, the principal CD44 ligand, is a nonsulfated glycosaminoglycan composed of repeats of D-glucuronic acid and *N*-acetyl-D-glucosamine disaccharide units and it is an important component of the extracellular matrix [13]. Hyaluronan may operate during the development of the macroangiopathy because it occurs in abnormal and large amounts in the vessel wall among people with diabetes mellitus [10]. It also plays a role in cellular migration and proliferation of the cells, through interactions with its

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receptor, CD44 [14-16]. This receptor is induced on vascular smooth muscle cells (SMCs) after arterial wall injury and may mediate proliferation and migration into the tunica intima [17,18]. Putatively, large amounts of HA may increase the presence of arterial SMCs in tunica intima, where they seem to be prerequisite for the development of atherosclerotic plaque. The accumulation and consequences of HA in the vessels of diabetic patients remains, however, unexplained [10].

CD44 is a broadly distributed cell surface glycoprotein that mediates cell-cell adhesion and cell-matrix interactions [19]. Among its many physiological functions, CD44 has been implicated in extracellular matrix binding [20], cell migration [17,18], leukocyte activation [21,22], and lymphocyte homing [23]. This HA receptor is encoded by a single gene, but is expressed as multiple isoforms of different molecular sizes (80-250 kd). The genomic organization involves 20 exons. Ten exons encode the CD44 sequence that is expressed generally, but one or more of 10 variable exons can be inserted by alternative splicing [24,25]. The most common form of CD44 referred to as standard CD44, has an apparent molecular weight of 80 to 90 kd and does not contain any of the variable exons [25].

The present study investigates the relationship between some of the components in the metabolic disorders of diabetes and the CD44 quantity and function on humane arterial SMCs. Thus, the influence of various concentrations of glucose, insulin, insulin-like growth factor I (IGF-I), and human growth hormone (hGH) on the expression of standard CD44 and the variant isoforms, CD44v3 and CD44v6, on arterial SMCs is evaluated. Furthermore, the purpose of the study was to examine the effect of glucose, insulin, IGF-I, and hGH on the functional role of CD44 in SMC migration.

2. Materials and methods

2.1. Materials

Rat anti-CD44 monoclonal antibody (mAb; clone: 1M7.8.1) used in the enzyme-linked immunosorbent assay (ELISA) was purchased from Zymed Laboratories Inc (South San Francisco, Calif). The antihuman homing-associated cell-adhesion molecule (CD44) used in the migration study was obtained from Pierce Biotechnology Inc (Rockford, Ill) [26]. Monoclonal antihuman CD44v3 (clone: 3G5) and antihuman CD44v6 (clone: 2F10) were purchased from R&D Systems (Minneapolis, Minn). The secondary antibodies biotinylated antirat IgG and biotinylated antimouse IgG were purchased from Vector Laboratories (Burlingame, Calif). Purified HA from human umbilical cord was obtained from Seikagaku Corporation (Tokyo, Japan). The molecular weight of HA was approximately 800 to 1200 kd according to the manufacturer's specifications. 2-(4′-Hydroxyphenylazo)benzoic acid and avidin were purchased from Pierce (Rockford, Ill).

2.2. Cell cultures

Arterial SMCs were obtained from normal human aortas. Explants of the tunica media were incubated in minimal essential medium (MEM) containing 5% newborn calf serum and 5% human serum, 50 μg/mL gentamycin, 2 mmol/L L-glutamine, 2 μg/mL fungilin, and 100 μg/mL ampicillin (Gibco BRL Div. of Invitrogen, Gaithersburg, Md). The cells were maintained at 37°C in an atmosphere of 5% CO_2/95% atmospheric air for 4 to 6 weeks. The cells were trypsinized, resuspended, and incubated in culture flasks until they reached confluency. Third-passage cells were used in the experiments. The Local Ethical Committee approves the experiments.

2.3. Experimental conditions

After reaching confluence, the cells were trypsinized, counted, and resuspended (60 000-80 000 cells per milliliter) in MEM containing 5% newborn calf serum and 5% human serum and plated in 96-well tissue culture plates. On day 2, the medium was changed. The cells were cultured in MEM containing 0.1% human serum albumin (HSA) for 24 hours before various test substances were added. The substances included 0, 50, 100, 200, and 1000 μU/mL insulin (Actrapid, Novo Nordisk, Denmark); 0, 2, 4, 8, and 16 ng/mL recombinant hGH (Norditropin, Novo Nordisk, Denmark); 5.5, 8, and 16 mmol/L glucose; and 0, 0.6, 6, and 60 μmol/L IGF-I (Pharmacia, Sweden).

2.4. Migration assay

Smooth muscle cells were plated (10 μL cells at a density of 3×10^6 cells per milliliter is added to 500 μL MEM in the well) into stainless-steel fences placed in the center of the well using 24-well plates [27]. Smooth muscle cells were grown in MEM containing 10% calf serum. After 24 hours, the fences were removed. The SMCs were treated for 2 hours with 20 μg/mL mitomycin C (Sigma Aldrich, St Louis, Mo) to prevent proliferation. Medium was then removed, and cells were placed in MEM containing 2.5% newborn calf serum including various test substances: 0, 50, 200, and 1000 μU/mL insulin; 0, 2, 8, and 16 ng/mL hGH; 5.5, 8, and 16 mmol/L glucose; or 0, 0.6, 6, and 60 μmol/L IGF-I. Furthermore, anti-CD44 mAb (1:1000) or HA (10 μg/mL) was added to half of the wells. Cells were refed at day 3, and after 6 days in culture, washed with PBS, fixed in 4% formalin, and stained with methylene blue. Cell surface area was determined by point count in a light microscope and the radial migration was calculated as square millimeters. Migration rates for the experimental groups were referenced to the control group (SMCs cultured alone) and expressed as percent of control.

2.5. ELISA procedure

A modified ELISA procedure was used to measure cellular CD44 content. Cells were grown in 96-well plates and exposed to various test substances as indicated above

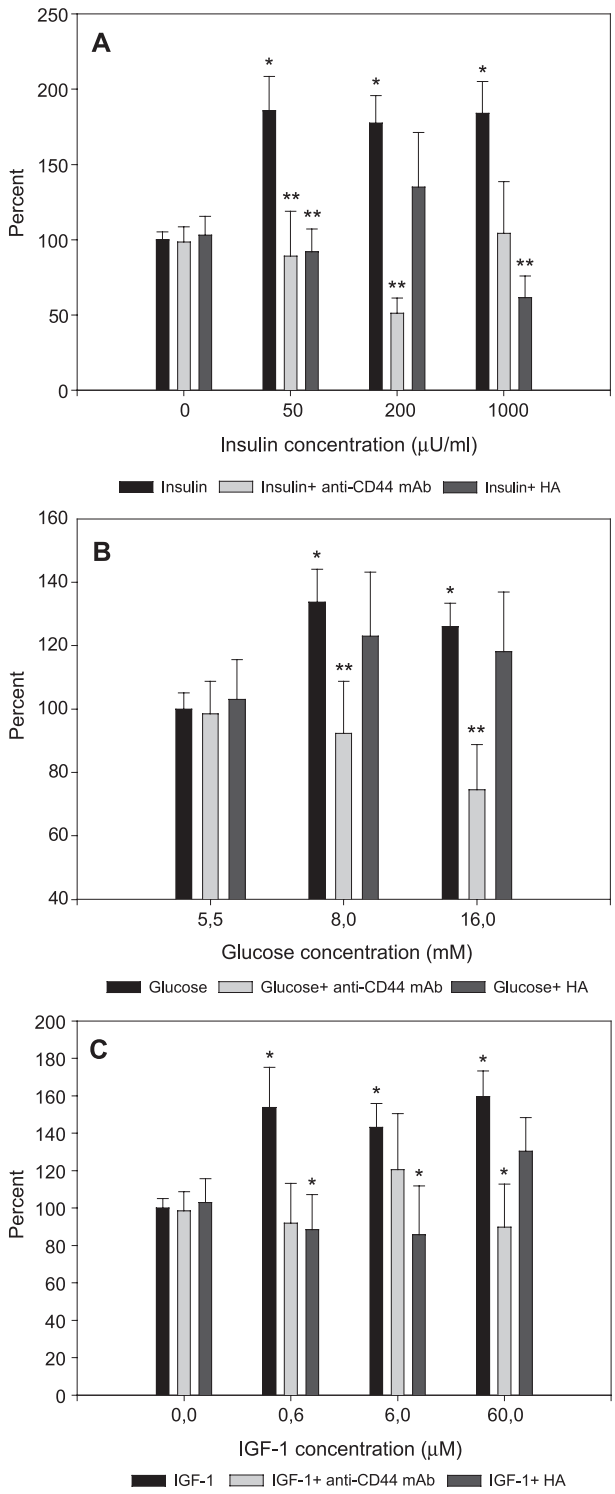

Fig. 1. A, Migration of SMCs cultured with insulin. The black bars illustrate the migratory effect of insulin (50-1000 μU/mL). The light gray bars show the inhibitory effect of anti-CD44 mAb (1:1000) on the insulin-stimulated migration, and the dark gray bars represents the effect of HA (10 μg/mL) on the insulin stimulated migration. Values are means ± SEM (number of cultures = 5-14). *2P < .05, compared with the black control bar. **2P < .05, compared within the same concentrations. B, Migration of SMCs cultured with glucose. The black bars illustrate the migratory effect of glucose (5.5-16 mmol/L). The light gray bars show the inhibitory effect of anti-CD44 mAb (1:1000) on the glucose-stimulated migration. The dark gray bars represents the effect of HA (10 μg/mL) on the glucose-stimulated migration. Values are means ± SEM (number of cultures = 5-20). Control was 5.5 mmol/L. *2P < .05, compared with the black control bar. **2P < .05, compared within the same concentrations. C, Migration of SMCs cultured with IGF-I. The black bars illustrate the migratory effect of IGF-I (0.6-60 μmol/L). The light gray bars show the inhibitory effect of anti-CD44 mAb (1:1000) on the IGF-I–stimulated migration, and the dark gray bars represents the effect of HA (10 μg/mL) on the IGF-I–stimulated migration. Values are means ± SEM (number of cultures = 5-15). *2P < .05, compared with the black control bar. **2P < .05, compared within the same concentrations.

for 24 hours, washed 3 times in PBS, air-dried, and stored at 4°C. Dried cells were rehydrated and blocked in PBS containing 0.1% bovine serum albumin (BSA) and 0.05% Tween for 20 minutes and then washed once in washing buffer (PBS containing 0.1% BSA and 0.05% Tween). The cells were then incubated for 2 hours at room temperature with either a mAb against CD44 diluted 1:4000 in washing buffer or mAbs against the isoforms CD44v3 and CD44v6 diluted 1:1000 in washing buffer. After 3 washes, the cells were incubated for 1 hour with biotinylated secondary antibodies diluted in washing buffer: biotinylated antirat IgG diluted 1:20 000 for CD44 measurements and biotinylated antimouse IgG diluted 1:10 000 for the CD44v3 and CD44v6 measurements. The cells were washed 3 times and incubated for 30 minutes with streptavidin horseradish peroxidase diluted 1:50 000, followed by 5 washes in washing buffer. Subsequently, the cells were colored using 100 μL tetramethylbenzidine substrate solution. The reaction was stopped by adding 150 μL of 0.18 mol/L H_2SO_4. Absorbance was read at 450 nm in an ELISA reader. Negative controls were preformed without incubation with the primary antibody.

2.6. HA-binding assay

Biotinylated HA (bHA) was prepared according to published methods [28,29]. The degree of biotinylation of the prepared bHA was determined spectrophotometrically to be 5.2 mol biotin per 1 mol HA (ë = 500 nm, using ImmunoPureR HABA and ImmunoPureR Avidin, Pierce Laboratories, Inc). Furthermore, total glucuronic acid content was determined with a carbazole reaction [30]. Cells were grown in 96-well plates and exposed to various test substances for 24 hours and washed 3 times in PBS, air-dried, and stored at 4°C. Dried cells were blocked in PBS containing 0.1% BSA and 0.05% Tween for 20 minutes and then washed once in washing buffer. The cells were then incubated for 1 hour with bHA (50 μg/mL). After 3 washes, the cells were incubated in streptavidin horseradish peroxidase and colored as in the ELISA procedure.

2.7. Western blot analysis

Cells were mixed with PBS containing 5 μL/mL protease inhibitors (0.86 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, and 0.25 mmol/L *N*-ethylmaleimide)

at 4°C and centrifuged at 10 000 rpm for 3 minutes. Pellets were resuspended in nonreducing sample buffer, boiled, and resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 4% to 12% Tris-glycine gel. Proteins were transferred to a polyvinylidine fluoride membrane at 400 mA for 60 minutes at 22°C, then blocked overnight at 4°C in PBS containing 5% nonfat milk and 0.05% Tween. The following day, the membrane was incubated for 1 to 2 hours at room temperature with rat anti-CD44 diluted 1:200, then the membrane was incubated with the secondary antibody biotinylated antirat IgG diluted 1:2000 and finally incubated for 30 minutes in streptavidin horseradish peroxidase diluted 1:50 000. Between the different incubations, the membrane was washed 3 times in PBS. CD44 was visualized by chemiluminescence Reagent Plus (Perkin-Elmer Life and Analytical Sciences, Boston, Mass). A negative control was preformed without incubation with the primary antibody.

2.8. Polymerase chain reaction analysis of reverse-transcribed CD44 messenger RNAs

Cells were grown in 6-well plates and cultured for 6 hours in MEM containing 0.1% HAS including the following test substances: 1000 μU/mL insulin, 16 mmol/L glucose, 60 μmol/L IGF-I, or 16 ng/mL hGH. Total RNA was extracted from the cells using 500 μL Trizol reagent (Gibco BRL) for each well, according to the instructions from the manufacturer. The amount and quality of the isolated RNA was determined by measuring the absorbance at 260 and 280 nm. From each sample, 1 μg RNA was subjected to reverse transcription in 30 μL of reaction mixture containing 50 mmol/L Tris (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 0.5 mmol/L of each deoxyribonucleotide triphosphate, 50 pmol random hexamer, and 200 U of Moloney murine leukemia virus reverse transcription (Gibco BRL). The samples were incubated for 75 minutes at 37°C, followed by incubation for 5 minutes at 95°C.

Polymerase chain reaction was subsequently performed using 2 μL of reverse-transcribed material in a reaction mixture of 20 μL containing 10 mmol/L Tris (pH 8.8), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L of each deoxyribonucleotide triphosphate, 1 U DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland), and 500 pmol of each of the primers. The following primers were used: CD44 standard: 5′ CD44 std: 5′-AGTCACAGACCTGCCCAATGCCTTT-3′ and 3′ CD44 std: 5′-TTTGCTCCACCTTCTTGACTCCCATG-3′; Cd44v3: 3′ CD44v3: 5′-GGTGTCTGTCTCTTTCATCTTCATTTTCTTCATTT-3′ (exon v3). β-Actin primers were used as control. Amplification of complementary DNA was performed in a Hybaid Thermocycler with 2 initial cycles (95°C for 60 s, 50°C for 60 seconds, 73°C for 1.30 minutes) followed by 35 cycles (94°C for 45 seconds, 50°C for 45 seconds, 73°C for 1.10 minutes). For the final cycle, the sample were incubated for 2 minutes at 73°C. The polymerase chain reaction products were separated by gel electrophoresis in a 2% agarose gel containing ethidium bromide. The intensity of visualized bands was measured in arbitrary absorbance units with a Bio-Rad UV-gel camera and used as an estimate of messenger RNA (mRNA) amount. Negative controls without RNA did not give rise to DNA amplification (results not shown).

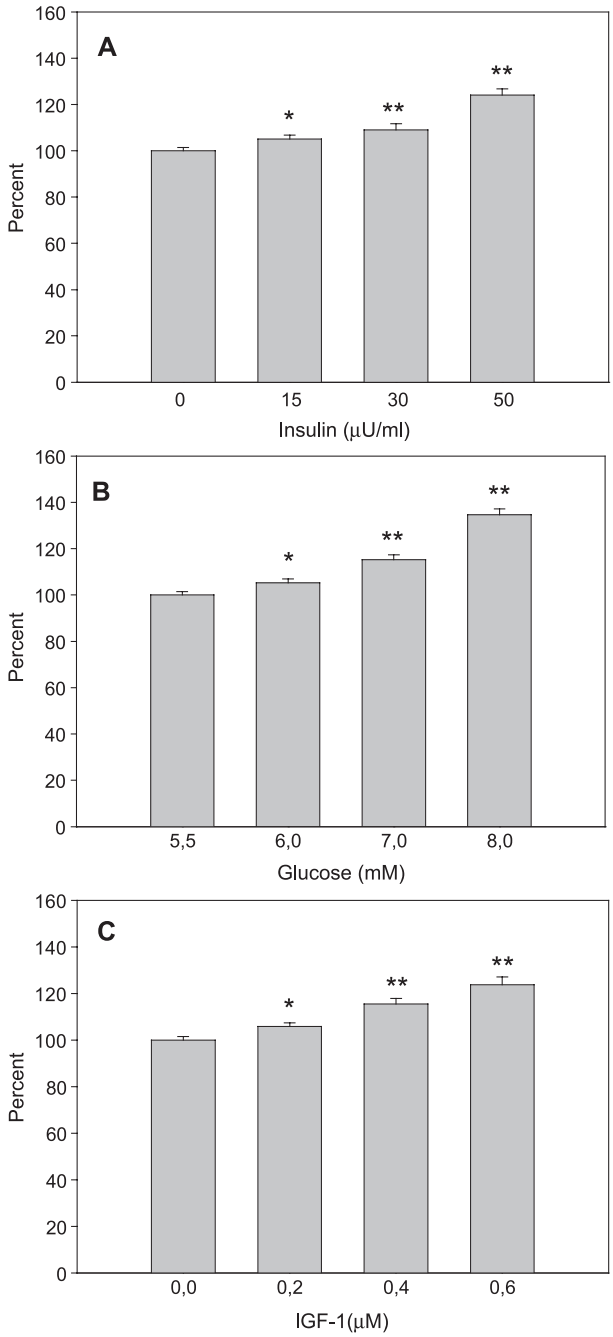

Fig. 2. A, Migration of SMCs cultured with smaller concentrations of insulin. Values are means ± SEM (number of cultures = 8-12). *2P < .04, **2P < .005. B, Migration of SMCs cultured with smaller concentrations of glucose. Values are means ± SEM (number of cultures = 10-11). *2P < .03, **2P < .00001. C, Migration of SMCs cultured with smaller concentrations of IGF-I. Values are means ± SEM (number of cultures = 6-10). *2P < .03, **2P < .00005.

Table 1
Expression of CD44 and CD44v3 and HA binding after incubation of the SMCs with insulin, glucose, IGF-I, or hGH

Insulin	Control	50 μU/mL	100 μU/mL	200 μU/mL	1000 μU/mL
CD44	100.00 ± 2.5	100.96 ± 2.6	97.75 ± 1.6	103.88 ± 2.4	108.42 ± 2.6*
CD44v3	100.00 ± 1.7	96.68 ± 1.2	98.51 ± 1.2	97.00 ± 1.5	101.73 ± 1.8
HA binding	100.00 ± 2.7	98.81 ± 2.9	110.30 ± 3.9*	110.82 ± 3.0*	116.18 ± 4.0*
Glucose	**Control**	**8 mmol/L**	**16 mmol/L**		
CD44	100.00 ± 1.4	98.78 ± 1.1	99.74 ± 1.2		
CD44v3	100.00 ± 1.1	92.39 ± 1.9*	93.51 ± 1.4*		
HA binding	100.00 ± 4.5	108.42 ± 3.4	107.68 ± 3.5		
IGF-I	**Control**	**0.6 μmol/L**	**6 μmol/L**	**60 μmol/L**	
CD44	100.00 ± 2.5	90.85 ± 1.9	93.91 ± 2.8	94.50 ± 2.7	
CD44v3	100.00 ± 1.56	94.09 ± 1.3*	88.19 ± 2.1*	98.12 ± 1.41	
HA binding	100.00 ± 2.5	110.76 ± 4.0	104.97 ± 3.6	99.02 ± 3.7	
hGH	**Control**	**2 ng/mL**	**4 ng/mL**	**8 ng/mL**	**16 ng/mL**
CD44	100.00 ± 1.6	93.83 ± 1.1*	95.84 ± 1.5	99.83 ± 1.8	106.40 ± 1.7*
CD44v3	100.00 ± 3.1	96.14 ± 1.3	92.71 ± 2.5	100.02 ± 1.8	105.30 ± 1.4
HA binding	100.00 ± 3.2	104.17 ± 3.2	101.34 ± 3.4	96.33 ± 2.4	100.95 ± 3.2

Data are presented as means ± SEM (number of cultures = 12-40).
* $2P < .05$.

2.9. Statistical methods

The results are expressed as percent of control values presented as means ± SEM. Statistical evaluation was performed with ANOVA and the Student *t* test. A $2P$ value less than .05 were accepted as statistically significant.

3. Results

3.1. Migration assay

Data showed that insulin augmented the migration ($2P < .01$) (Fig. 1A). Addition of the mAb against CD44 significantly reduced the insulin-elevated migration to control level at 50 and 200 μU/mL insulin ($2P < .01$). The addition of HA down-regulated the insulin-stimulated migration at 50 and 1000 μU/mL ($2P < .01$) (Fig. 1A). Glucose stimulates significantly SMC migration at concentrations of 8 and 16 mmol/L ($2P < .01$). Addition of anti-CD44 mAb reversed the glucose-stimulated migration at 8 and 16 mmol/L glucose ($2P < .05$). Supplementation of HA did not change the glucose-stimulated migration (Fig. 1B). Various amounts of hGH revealed no significant change in SMC migration. IGF-I promotes the migration ($2P < .01$), and supplementation of anti-CD44 mAb only decreased the migration at 60 μmol/L IGF-I ($2P < .03$). Hyaluronan significantly reduced the IGF-I–stimulated migration of SMCs at 0.6 and 6 μmol/L IGF-I ($2P < .05$). There was no significant decrease when HA was added simultaneous with 60 μmol/L IGF-I (Fig. 1C). In Fig. 1A, B, and C, the maximal effect is achieved at the lowest concentration of insulin, glucose, and IGF-I used. Therefore, the smaller concentrations were investigated. As shown in Fig. 2A, B, and C, smaller concentrations of insulin, glucose, and IGF-I demonstrates a significant dose-dependent increase in migration.

3.2. ELISA procedure

A modified ELISA procedure was applied to measure cellular CD44 content using monoclonal antirat CD44 as primary antibody (Table 1). The data showed that 1000 μU/mL insulin resulted in a significant but small increase in CD44 expression ($2P < .03$). Human growth hormone significantly elevated the CD44 amount only at a high concentration of 16 ng/mL hGH ($2P < .01$), whereas lower

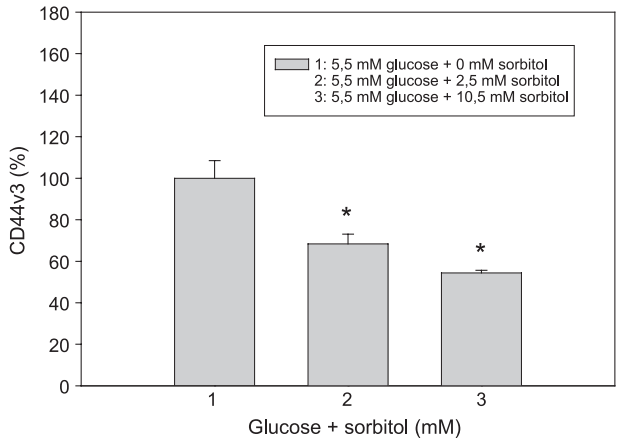

Fig. 3. Osmotic control experiment. The effects of glucose + sorbitol (5.5 mmol/L glucose + 2.5 or 10.5 mmol/L sorbitol) on CD44v3 expression on SMCs in culture. Data are presented as means ± SEM (number of cultures = 9-10). *$2P < .004$.

concentrations of 2 ng/mL hGH showed a decrease ($2P < .01$). It was not possible to demonstrate any effect of glucose and IGF-I on the CD44 expression.

Measurements were also performed using the antibodies against the isoforms CD44v3 and CD44v6. The results obtained showed that CD44v6 was not present, whereas Cd44v3 is expressed on human vascular SMCs. The amount of CD44v3 decreased significantly ($2P < .01$) at 8 and 16 mmol/L glucose. Incubation with 8 and 16 mmol/L carbohydrate (5.5 mmol/L glucose + 2.5 or 10.5 mmol/L sorbitol) resulted in a significantly reduction of the expression of CD44v3 ($2P < .004$) (Fig. 3). At lower concentrations (0.6 and 6 μmol/L), IGF-I CD44v3 expression decreased significantly ($2P < .01$). Various amounts of insulin and hGH revealed no change in CD44v3 expression.

3.3. Effects of glucose, insulin, IGF-I, or hGH on CD44 mRNA expression

Polymerase chain reaction analysis of mRNA from SMCs treated with high concentrations of glucose (16 mmol/L), insulin (1000 μU/mL), IGF-I (60 μmol/L), or hGH (16 ng/mL) was performed. The expression of CD44 std mRNA showed no detectable difference after incubation with each of the 4 metabolic components. CD44v3 mRNA level was significantly decreased after incubation with glucose and hGH, whereas incubation with insulin and IGF-I showed no detectable difference in CD44v3 mRNA expression. Data are expressed as means of CD44 std/β-actin $\times$ 100 or CD44v3/β-actin $\times$ 100 $\pm$ SEM (Table 2). Note that the data are relative numbers.

3.4. Western blot analysis

Western blot analysis of cell membrane preparations was performed. As shown in Fig. 4, vascular SMCs express 1 predominant band of 85 kd, corresponding to the apparent molecular weight of CD44 std. Furthermore, the SMCs express a weaker triplet around 66 kd and a weak band of approximately 180 kd, suggesting the presence of some isoforms on vascular SMCs. The band of 180 kd might be because of CD44v3. The molecular weight of CD44v3 has previously been reported to be 140 kd; however, the molecular weight could vary in relation to posttranslational modifications [31].

Table 2
Reverse transcription polymerase chain reaction amplification of RNA from SMCs treated with insulin, glucose, IGF-I, or hGH, using primers specific for CD44 std and CD44v3

	CD44	CD44v3
Control	66.13 $\pm$ 2.60	133.57 $\pm$ 5.50
Insulin (1000 μU/mL)	66.45 $\pm$ 2.65	124.39 $\pm$ 7.40
Glucose (16 mmol/L)	58.74 $\pm$ 3.08	100.12 $\pm$ 6.52*
IGF-I (60 μmol/L)	73.22 $\pm$ 3.11	145.27 $\pm$ 3.81
hGH (16 ng/mL)	64.67 $\pm$ 2.68	113.08 $\pm$ 4.86*

Data are presented as means of CD44 std/β-actin $\times$ 100 or CD44v3/β-actin $\times$ 100 $\pm$ SEM (number of cultures = 5-12).
* $2P < .05$.

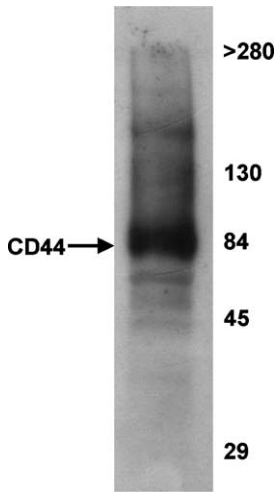

Fig. 4. Identification of CD44 on human arterial SMCs. Cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing conditions followed by Western blotting. Molecular weight markers (in kilodaltons) are indicated.

3.5. HA-binding assay

In the bHA-binding assay, a dose-dependent increase in bHA binding was observed when the cultures were supplemented with 100, 200, and 1000 μU/mL insulin ($2P < .03$). It was not possible to demonstrate any effect of glucose, IGF-I, and hGH on bHA binding (Table 1).

4. Discussion

Normally, vascular SMCs reside in the tunica media as a contractile quiescent phenotype, but after wall injury, these cells are transformed to a proliferative migratory phenotype that secretes abundant extracellular matrix [32,33]. It is known that CD44 receptor is induced on SMCs after wall injury and that it may mediate their migration into the tunica intima [17]. Furthermore, a number of publications have found that HA is up-regulated in atherosclerotic lesions from human beings [34-36]. In the present study, the effect of various concentrations of glucose, insulin, IGF-I, and hGH on SMC migration were examined and it was demonstrated that glucose, insulin, and IGF-I stimulated the migration of SMCs significantly. Monoclonal antibodies against the CD44 receptor was able to decrease this increased migration to control level at most concentrations, which confirms that CD44 receptor plays a role in SMC migration. Addition of HA decreased the insulin- and IGF-I–stimulated migration of SMCs but it has no effect on the glucose-stimulated migration. It should be emphasized that the HA used in the present study was a high–molecular-weight form, which has been shown to contribute to the maintenance of the contractile quiescent phenotype of SMCs [25,37]. It is important to note that it is not possible to compare these in vitro effects with the situation in vivo, where the concentration of HA is lower 10 and the molecular forms may differ. Furthermore, the SMCs showed

a tendency to loosen from the bottom of the culture plate when HA was added. A role of HA in cell detachment has previously been suggested because exogenously added HA stimulated detachment of BALB 3T3 cells [38]. CD44-HA interactions mediate cell aggregation via cell-matrix cross-bridging. However, when the HA levels reaches saturation of the available CD44 receptor sites, the cells disaggregate [39]. Human growth hormone revealed no significant change in SMC migration.

Subsequently, the CD44 protein and mRNA expression level after incubation with glucose, insulin, IGF-I, and hGH were investigated to verify whether the demonstrated stimulation of SMC migration after metabolic manipulation is a result of increased CD44 expression. The highest concentration of insulin resulted in a significant but small increased CD44 expression, whereas CD44 content was reduced at 2 ng/mL hGH and elevated at 16 ng/mL hGH. The arterial SMCs express IGF-I receptor [40], but no effect of IGF-I was demonstrated on CD44 amount. Glucose showed no effect on the expression of CD44 receptor.

Glucose and IGF-I were able to decrease protein expression of the variant isoform CD44v3 receptor, whereas insulin and hGH showed no effect on the amount of CD44v3 receptor. Given that CD44v3 expression is decreased at high glucose level, SMCs were incubated with 8 and 16 mmol/L sorbitol as a control for the osmotic effect of glucose. The data showed that the glucose effect could be ascribed to an osmotic influence. CD44v3 isoforms uniquely contains Ser/Gly sites that direct *O*-glycosylation with heparan sulfate and chondroitin sulfate, which generates CD44v3 heparan sulfate proteoglycan forms [41,42]. It has been shown that these modified CD44v3 isoforms bind growth factors such as vascular endothelial growth factor, heparin-binding epidermal growth factor (HB-EGF), and the IGF family [43-45]. Thus, the decreased amount of CD44v3 isoforms after incubation with diabeticlike concentrations of glucose and IGF-I may influence the action of these growth factors. Our results showed that the variant isoform CD44v6 was not present at human arterial SMCs. In contrast, Jain et al [17] have shown that CD44v6 is expressed on rat aortic SMCs indicating that there seem to be differences between rat and human arterial SMCs with respect to expression of CD44v6 receptor.

We were not able to detect any difference in expression of CD44 std mRNA with any of the metabolic components, suggesting that the small difference in CD44 protein amount may be because of posttranslational regulation. For the CD44v3 isoform, there was more resemblance with protein measurements, because a decreased level was shown after incubation with glucose both at protein and mRNA level. It should be emphasized that time studies were not performed. The SMCs were cultured with the metabolic components for 6 hours before RNA was extracted. Therefore, changes in mRNA expression level might have happened at a different time. However, it appears that the metabolic components only are able to induce small alterations in CD44 expression. As mentioned, SMCs are transformed to a proliferative migratory phenotype after vessel wall injury, with an increased CD44 expression [17,32,46]. This is in contrast to the situation in our study where confluent SMCs, which probably possess the contractile quiescent phenotype, are analyzed. Thus, methodical aspects might explain the discrepancy between the small changes in CD44 expression and the more persistent effects on SMC migration after metabolic manipulation. On the other hand, recent evidence suggests that activation of the CD44 receptor can be achieved by posttranslational mechanisms independent of CD44 amount [47]. Therefore, the observed difference might be a question of posttranslational CD44 activation rather than CD44 expression levels. However, activation studies with, for instance, CD44-specific mAbs or with inducers such as phorbol ester [48] were not performed in this study.

Binding of HA was increased when the SMCs were incubated with insulin, which correlates with the fact that insulin was able to elevate the CD44 expression; however, glucose, IGF-I, and hGH showed no effect on the binding of HA. Experimentally induced diabetes has been shown to induce a significant elevation in serum HA concentration, which can be reduced by insulin treatment [10,49]. Furthermore, in vitro studies with arterial SMCs have revealed that insulin is able to reduce the amount of synthesized HA [50]. Thus, it is possible that the produced HA might interfere with the binding capacity analyzed in the present study. However, the results support that insulin may have a significant influence on the development of large vessel disease. The fact that glucose, IGF-I, and hGH showed no effect on the binding of HA does not correlate with the CD44 expression studies, where hGH was able to decrease CD44 expression and where CD44v3 expression was decreased after incubation with glucose and IGF-I. Although the differences may be explained by different sensitivities of these 2 techniques or may be because of the fact that the HA used in this study was a high–molecular-weight form with multiple binding sites along its length.

In conclusion, our results indicate that metabolic and hormonal factors exhibit influence on SMC CD44 expression and particularly on the migration. It is not possible, of course, to draw conclusions between the results obtained and the situation prevailing in the large vessels of patients with diabetes. The present study is related to our earlier reports of vascular disease in diabetes mellitus both to investigations of the amount of HA in aortic tissue and to in vitro analysis of arterial SMC production of HA [10,50]. Together, these data are compatible with the notion that HA-CD44 interactions may play important roles in the development of large vessel disease in diabetes.

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