Vascular Cell Adhesion Molecule-1 Gene Expression during Human Smooth Muscle Cell Differentiation Is Independent of NF-κB Activation*

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Vascular cell adhesion molecule-1 (VCAM-1) gene expression in cytokine-activated cells depends on two κB elements. Since VCAM-1 expression appears developmentally regulated and cytokine-inducible in smooth muscle cells (SMCs), we have studied the role of NF-κB in differentiated SMC VCAM-1 expression. Confluent SMCs were cultured either in a serum-free medium in order to induce differentiation, or in medium with serum, stimulated or not by tumor necrosis factor α (TNF-α). The expression of smooth muscle myosin heavy chain, a SMC marker, and VCAM-1 was induced concomitantly in serum-free medium, whereas only VCAM-1 expression was induced by cytokine treatment. We showed that the p50 and p65 subunits of NF-κB were localized in the cytoplasm of differentiating SMCs, whereas they were translocated into the nucleus of TNF-α-activated SMCs. Electrophoretic mobility shift assays with VCAM-1 gene κB elements failed to detect any induction of DNA-protein complex with nuclear extracts of differentiating SMCs in contrast to the cytokine-activated SMC nuclear extracts. Furthermore, VCAM-1 mRNA induction was inhibited in TNF-α-stimulated SMCs, but not in differentiating SMCs, by pyrrolidine dithiocarbamate, an inhibitor of NF-κB protein activation. Taken together, these findings suggest that in contrast to TNF-α activation, NF-κB is not involved in VCAM-1 gene expression during SMC differentiation.

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1 The abbreviations used are: VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4; SMC, smooth muscle cell; sm-MHC, smooth muscle-myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; bp, base pairs; PDGF, platelet-derived growth factor; TGF, transforming growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; IGF, insulin-like growth factor; IRF, interferon regulatory factor.

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Role of NF-κB in SMC VCAM-1 Gene Expression

In contrast with the strong inhibition of TNF-α-mediated VCAM-1 mRNA induction during SMC differentiation, NF-κB was not involved in VCAM-1 gene expression during SMC differentiation. MATERIALS AND METHODS

Cytokines and Antibodies—Recombinant human TNF-α was provided by Genzyme, Inc. (Cambridge, MA). Murine monoclonal antibody directed against human VCAM-1 (monoclonal antibody 1G11, diluted 1:50) was produced from Immunotech. Anti-human smooth muscle myosin heavy chain (sm-MHC) monoclonal antibody was kindly provided by Dr. M. Glukhova (25) (diluted 1:100). Rabbit polyclonal antibodies to NF-κB family member p50 and p65 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; diluted 1:200).

Cells—Smooth muscle cells were isolated from media of human thoracic aortic smooth muscle cultures to NF-κB activity (23, 24), did not modify VCAM-1 mRNA induction during SMC differentiation, in contrast with the strong inhibition of TNF-α-induced expression of VCAM-1. These results indicate that NF-κB family members are not involved in VCAM-1 gene expression during SMC differentiation.

RNA Analysis—Total cellular RNA was prepared using a single-step acid guanidium isothiocyanate/phenol/chloroform extraction method (29): first-strand cDNA synthesis by reverse transcription (30) using 1 μg of total RNA and 20 μg/ml oligodeoxynucleotides and 200 units of MMLV reverse transcriptase (Life Technologies, Inc.) with incubation for 1 h at 42 °C. Specific primers for G3PDH, VCAM-1, and sm-MHC were used as described previously (8) to amplify regions of cDNA copies from total RNA.

Oligonucleotide Sequences—In order to prepare double-stranded oligonucleotides containing the upstream κB elements of human VCAM-1 gene, synthetic complementary single-stranded oligonucleotides were annealed and then purified by polyacrylamide gel electrophoresis. Gel-purified double-stranded oligonucleotides were labeled on their 5′ ends with [γ-32P]ATP (5000 Ci/mmol) (Amersham Pharmacia Biotech) using T4 polynucleotide kinase and used as probes in Southern blotting (31). For hybridization, the DNA sequence determined by the dideoxy chain-termination method with modified T7 DNA polymerase. The VCAM-1 and sm-MHC cDNA fragments were labeled by random priming with the kit Ready-To-Go (Biolabs). Hybridization was done at 42 °C overnight. Filters were washed in 2× SSC, 10× Denhardt’s solution (1× Denhardt’s solution is 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, 0.02% BSA), 0.1% SDS, and sheared denatured salmon sperm DNA at 0.4 mg/ml. Filters were washed in 2× SSC for 10 min at room temperature, followed by two washes in 0.1× SSC for 30 min at 55 °C. Filters were exposed to Kodak XAR-5 film with an intensifying screen.

Preparation of Nuclear Extracts—Preparation of nuclear extracts was based on the methodologies described by Li and Schreiber (32, 33). The cultured cells (approximately 2×106 cells) were washed with cold PBS and harvested after trypsin treatment. The cells were centrifuged at 4,000 rpm for 10 min, and the cell pellet was resuspended in 800 μl of cold buffer A (10 mM HEPES, pH 8, 50 mM NaCl, 0.5 mM sucrose, 1 mM EDTA, pH 8, 0.5 mM spermidine, 0.15 mM spermine). The cells were allowed to swell on ice for 15 min, then 25 μl of a 10% solution of Nonidet P-40 was added to release the nuclei. Nuclei were recovered by centrifugation at 3,000 rpm for 10 min at 4 °C. The nuclear pellet was resuspended in 500 μl of buffer B (10 mM HEPES, pH 8, 50 mM NaCl, 25% glycerol, 0.1 mM EDTA, pH 8, 0.5 mM spermidine, 0.15 mM spermine) in the presence of protease inhibitors (pestatin, leupeptin, aprotonin, and chymostatin) at 5 μg/ml, chelating reagent (1 mM EDTA), and 1 mM dithiothreitol. The nuclear pellet was recovered by centrifugation, and nuclear proteins were extracted by incubation for 1 h at 4 °C in 100 μl of buffer C (the same as buffer B except 400 μl NaCl). The nuclear extract was centrifuged at 13,500 rpm for 40 min, and the supernatant was dialyzed three times against 250 ml of buffer D (20 mM HEPES, pH 8, 50 mM KCl, 20% glycerol, 0.2 mM EDTA, pH 8, 0.5 mM spermidine, and 1% Triton X-100) at 4 °C. Nuclear extracts were stored in aliquots at −80 °C. Protein concentration was measured by the Bradford assay (Bio-Rad kit) (34).

Electrophoretic Mobility Shift Assay— EMSA was performed with 25 μl of binding mixture containing 1 μg of double-stranded poly(dI-dC), 40 fmoles of 5′-labeled annealed κB oligonucleotides, 8 μg of nuclear extract in 12 mM HEPES, pH 8, 50 mM KCl, 12% glycerol, 1 mM MgCl2, 0.6 mM dithiothreitol, 0.12 mM EDTA, 1.2 mM benzamidine. Reactions were incubated at room temperature for 30 min, and DNA-protein complexes were analyzed in a Couter type XL flow cytometer.

Electrophoretic mobility shift assay (EMSA) failed to detect any induction of NF-κB activity during SMC differentiation. Addition of the antioxidant pyrrolidine dithiocarbamate (PDTC), inhibitor of NF-κB activity (23, 24), did not modify VCAM-1 mRNA induction during SMC differentiation. Addition of the antioxidant pyrrolidine dithiocarbamate (PDTC), inhibitor of NF-κB activity (23, 24), did not modify VCAM-1 mRNA induction during SMC differentiation.
were resolved by non-denaturing polyacrylamide electrophoresis in 5% acrylamide, employing 0.022 M Tris borate, 0.5 mM EDTA, pH 8, as buffer. Following electrophoresis, gels were dried and DNA-protein complexes were localized by autoradiography. Competition studies were performed by adding unlabeled double-stranded oligonucleotides to the binding reaction.

RESULTS

VCAM-1 Induction Model during SMC Differentiation—In order to determine the role of the NF-κB/Rel protein family in VCAM-1 expression in SMCs, we used an in vitro model of SMC phenotypic modulation. Concerning the modulation of differentiation process, we analyzed the protein and mRNA levels of sm-MHC, a marker of the fully differentiated phenotype of SMCs (36–38). In 6-day post-confluent cells, maintained in medium with serum, a faint immunostaining for sm-MHC was observed, suggesting a dedifferentiated phenotype (Fig. 1, S). When these post-confluent cells in medium with serum were stimulated for 24 h with TNF-α, the same low expression of sm-MHC was observed (Fig. 1, T), showing the lack of effect of TNF-α on SMC differentiation. On the other hand, when the confluent cells were cultured for 6 days in the defined medium without serum, a strong immunostaining for sm-MHC was noted, indicative of redifferentiation (Fig. 1, D). Analysis of mRNA content by RT-PCR and Southern transfer revealed a low level of sm-MHC mRNA in cells cultured in medium with serum that was stimulated or not stimulated with TNF-α (Fig. 2). In SMCs cultured in defined serum-free medium, a high level of sm-MHC mRNA was detected (Fig. 1, D). This suggested that VCAM-1 was induced in two different cell populations, in TNF-α-stimulated undifferentiated SMCs and in redifferentiated SMCs.

Localization of NF-κB/Rel Proteins during VCAM-1 Induction on SMCs—In order to determine the cellular distribution of NF-κB/Rel proteins in SMCs, the immunostaining of the p50 and p65 subunits of NF-κB complex was studied in SMCs cultured under different conditions at 6 days (Fig. 3).

Analysis of specific probes was analyzed in the three conditions.

TABLE I
VCAM-1 protein expression in the in vitro model of SMC redifferentiation

| Condition                        | Corrected mean fluorescence | Normalized |
|----------------------------------|-----------------------------|------------|
| Serum                            | 48.5                        | 1          |
| TNF-α                            | 132.9                       | 2.74       |
| Defined serum-free medium         | 112.5                       | 2.32       |

Human undifferentiated serum-cultured SMCs (Serum), undifferentiated TNF-α-stimulated SMCs (TNF-α), and redifferentiated SMCs (Defined serum-free medium) were immunolabeled with anti-VCAM-1 monoclonal antibody (1G11). A fluorescent secondary antibody was applied, and 5,000 cells were analyzed by flow cytometry. Mean fluorescence obtained with no antibody was subtracted from the values obtained with specific antibody. Values are means of three independent experiments.

FIG. 1. sm-MHC protein expression in an in vitro model of SMC phenotypic modulation. Confluent second-passaged SMCs cultured in the presence of serum (S), stimulated by TNF-α (T), or maintained in defined medium without serum (D) were fixed with cold methanol and labeled immunocytochemically with a monoclonal antibody directed against human sm-MHC. A negative control was obtained by omitting the primary antibody (C, control). The nuclei were stained by Hemalan. Original magnification, × 20. The high expression of sm-MHC (D) indicated that the SMC redifferentiate in defined serum-free medium.

FIG. 2. Southern blot analysis of VCAM-1 and sm-MHC mRNA expression in SMC redifferentiation model. One μg of total RNA obtained from post-confluent SMCs cultured in medium supplemented with serum for 6 days, activated (T) or not (S) with 1000 units/ml TNF-α for 24 h, and from post-confluent SMCs cultured in a defined serum-free medium for 6 days (D) were used for RT-PCR analysis. Ten μl of DNA amplified product were analyzed in 1.5% agarose gel and transferred on nitrocellulose membrane. Hybridizations with specific end-labeled probes were used to ensure specificity of amplified products. DNA probes are described under “Materials and Methods.” G3PDH, used as internal control, VCAM-1, and sm-MHC mRNA expression were analyzed in the three conditions.
NF-κB proteins were translocated to the nucleus when SMCs were cultured in medium supplemented with serum or stimulated by TNF-α. This p50 and p65 protein translocation was not observed in redifferentiated SMCs.

In order to exclude an early involvement of NF-κB system in VCAM-1 expression during SMC redifferentiation process, we analyzed the time course of p50 Rel protein translocation in our culture model (Fig. 3b). As the result obtained after 6 days, we observed a weak p50 subunit staining into nucleus of SMCs maintained in serum. After TNF-α stimulation, a slight p50 nuclear staining was evident at 1 h (Fig. 3b, T). A maximal positive nucleus labeling was seen after 6 h of TNF-α stimulation and was maintained for 24 h (Fig. 3b, T). In redifferentiated SMCs, maintained in serum-free medium, we did not observe any nucleus staining but only a cytoplasmic labeling (Fig. 3b, D). These results seemed to confirm the non-involvement of NF-κB proteins in redifferentiated SMC VCAM-1 expression.

Characterization of Binding Activity of NF-κB/Rel Proteins during VCAM-1 Induction on SMCs—EMSA was employed in an attempt to find out whether nuclear proteins interact specifically with κB elements of the VCAM-1 gene promoter. Nuclear extracts of SMCs harvested in the three culture conditions described above were incubated with a double-stranded end-labeled oligonucleotide probe extending from position -88 to -56 of the human VCAM-1 promoter. As shown in Fig. 4, the nuclear extract of undifferentiated SMCs was not labeled, whereas addition of a 50-fold molar excess of an unrelated YY1 oligonucleotide or a 10–50-fold molar excess of an unrelated κB probe did not compete, indicating the specificity of this binding. Nuclear extracts from undifferentiated TNF-α-stimulated SMCs (T) formed two specific DNA-protein complexes (B1 and B2) with the probe. The complex was competed with a 10–50-fold molar excess of unlabeled κB oligonucleotide, whereas addition of a 50-fold molar excess of an unrelated YY1 oligonucleotide, or a 10–50-fold molar excess of the mutated κB probe did not compete, indicating the specificity of the two DNA-protein complexes. On the other hand, EMSA with nuclear proteins from differentiated SMCs (D) showed no specific DNA-protein complex. These results showed that the presence of serum was associated with the presence of the B1 complex, TNF-α stimulation inducing the appearance of the B2 complex. These complexes seemed to disappear in culture in defined serum-free medium, which induced redifferentiation.

Role of NF-κB/Rel Proteins in VCAM-1 Induction on SMCs—In an attempt to clarify the role of NF-κB/Rel proteins in the two conditions of VCAM-1 expression, after cytokine stimulation and during SMC differentiation, we examined whether the antioxidant PDTC, a blocker of NF-κB/Rel protein activation (20, 23, 35) interfered with VCAM-1 expression on SMCs. Since it has been shown that VCAM-1 mRNA in confluent SMCs is induced within 24 h after switching to a defined serum-free medium (8), the experiments with PDTC were performed at the onset of VCAM-1 mRNA induction during cytokine stimulation or SMC differentiation. As shown in Fig. 5, as expected, TNF-α treatment alone (lane 3) and switch to a defined serum-free medium (lane 5) induced a strong increase in VCAM-1 mRNA expression relative to that observed by...
treatment with serum (lane 1). In the presence of PDTC, we observed a complete inhibition of the cytokine-induced VCAM-1 mRNA expression (lane 4). A similar disappearance of the basal level of VCAM-1 mRNA expression in SMCs maintained in serum was observed in the presence of PDTC (lane 2). In contrast, induction of VCAM-1 mRNA on differentiating SMCs was unaffected (lane 6). These observations indicated that the involvement of NF-κB/Rel proteins in VCAM-1 expression depended on the status of the SMCs.

**DISCUSSION**

VCAM-1 is induced or up-regulated by proinflammatory cytokines such as TNF-α and interleukin-1β on many cellular components of the arterial wall, including endothelial cells, SMCs, and fibroblasts (3, 39–41). More recently, VCAM-1 has been reported to be inducible by TNF-α stimulation in a time- and dose-dependent manner on cultured SMCs (12). The pathophysiological significance in vivo of VCAM-1 expression on arterial SMC is unknown, although it may amplify inflammatory processes in pathological conditions such as atherosclerosis. However, as suggested by Rosen’s work on myocytes (4) and more recently by our own work in SMCs (8), homotypic cell-cell interactions mediated by VCAM-1/VLA-4 may participate in the regulation of cell differentiation. In a previous study, we demonstrated in an in vitro model of redifferentiation, SMCs reexpressed VCAM-1 and smooth muscle-specific markers during induction of differentiation (8). These observations suggested that VCAM-1 is expressed on the surface SMCs either after cytokine stimulation or during the process of differentiation. These two kinds of VCAM-1 up-regulation on SMCs were reproduced in the present experiments (cf. Fig. 1). However, the lack of induction of sm-MHC expression after TNF-α stimulation suggested different mechanisms for VCAM-1 induction on SMCs. Previous studies have shown that κB elements are involved in VCAM-1 gene induction by cytokines in endothelial cells, and for VCAM-1 expression during P19 cell differentiation into a neural pathway (6, 13). In an attempt to find out whether the VCAM-1 induction during SMC differentiation differed from the cytokine induction pathway, we analyzed the involvement of the NF-κB system in both processes.

Members of the NF-κB family are typically present in the cytoplasm bound to the inhibitory IκB proteins (21). Activation of NF-κB involves the phosphorylation and subsequently the degradation of inhibitory proteins (42), allowing NF-κB to translocate to the nucleus. The members of this family, which include p50, p52, p65, c-Rel, v-Rel, RelB, exist as homo- and heterodimers that bind to κB sites in the enhancer regions of a large number of target genes. As in many instances NF-κB binds as a heterodimer of a p50 in combination with p65 to stimulate gene expression (43), we analyzed the cellular distribution of p50 and p65 proteins in the different conditions of VCAM-1 expression. An intense staining of the nuclear compartment was observed for these two Rel proteins after TNF-α stimulation, the time course of NF-κB translocation into the nucleus showing an early nuclear staining for p50. In culture with serum, a weaker staining of the nucleus along with a labeling of the cytoplasm was observed. However, when the cells were cultivated for in defined serum-free medium allowing strong VCAM-1 expression and redifferentiation process, no immunostaining of the p50 and p65 subunits of the NF-κB/Rel family was observed in the nuclear compartment, whereas the cytoplasm was strongly labeled. The negative nuclear staining during the time-course experiments of NF-κB translocation into the nucleus excludes the hypothesis that NF-κB could be involved in VCAM-1 gene expression early in the differentiation stages. Cytokines such as TNF-α have been found to induce NF-κB activation in many cell types (22, 23). Moreover, growth factors that are contained in serum such as PDGF-BB, bFGF, EGF, and IGF-1 induce the translocation of p50 and p65 into the nuclei of SMCs, whereas growth inhibitors such as TGF-β and interferon-γ do not induce nuclear localization of these subunits of the NF-κB protein family (44). These findings are consistent with an involvement of the NF-κB complex in the TNF-α-induced VCAM-1 expression we observed. The absence of p50 and p65 subunit nuclear translocation during the redifferentiation induced by the switch to a defined serum-free medium indicated that VCAM-1 expression was independent of NF-κB complex activation in this case.

To directly address whether VCAM-1 expression in SMCs in different states of differentiation was associated with NF-κB activation and nuclear translocation, the binding activity of the κB sequence from the human VCAM-1 promoter for the different nuclear extracts was tested by EMSA. Specific DNA-protein complexes were observed on EMSA with nuclear extracts of TNF-α-treated and serum-treated SMCs. Only one DNA-protein complex, B1, was observed with serum-treated cells, and one faster migrating, additional complex, B2, with TNF-α-treated SMCs. Similar patterns have been reported by other workers: two NF-κB DNA-protein complexes in phorbol ester-activated Jurkat cells; the more rapidly migrating complex (B2) was composed of a p50 subunit and partial degradation products of p50, while the more slowly migrating complex (B1) contained not only these smaller proteins but also p65 and c-Rel (45). These two complexes were also observed in the same migration positions with nuclear extracts from rat aortic SMCs stimulated by various growth factors (44). In our experiments, complex B2 appeared only in human TNF-α-activated SMCs and may be associated with VCAM-1 induction. Complex B1, described after serum stimulation (44), did not appear to be in sufficient amount for VCAM-1 induction. In contrast, EMSA of nuclear extracts from differentiated SMCs, cultured for 6 days in defined serum-free medium, did not evidence any specific DNA-protein complex. Interestingly, Lehtinen et al. (46) reported that, after 48 h in low serum differentiation medium, the binding activity of NF-κB was very low in all myogenic cell lines tested (mouse C2C12 myocytes and rat L6 myocytes) and coincided with a most abundant nuclear staining of myogenin, a protein involved in terminal differentiation of skeletal muscle cells. In rat aortic smooth muscle cells (between third and sixth passage) starved with 0.1% fetal bovine serum for 3 days, only a faint B1 band was observed on EMSA by Obata et al. (44). In our experiments, the absence of the B1 band could have been due to the human origin of the cells or to the more differentiated state of the SMCs (8). The disappearance of a specific protein interacting with VCAM-1 gene promoter κB sites in nuclear extracts of serum-free medium cultured SMCs expressing VCAM-1, indicated that the NF-κB system was not in-

![Fig. 5. Antioxidant PDTC treatment prevents VCAM-1 mRNA induction in undifferentiated TNF-α-stimulated SMCs but not in redifferentiated SMCs.](image-url)
olved in its regulation during the process of differentiation.

In order to verify this idea, we analyzed the level of VCAM-1 mRNA expression after inhibition of NF-κB system activation by an antioxidant, PDTC. It has been shown that the activity of NF-κB as transcriptional factor is regulated by changes in the redox state of the cell (47). In human vascular endothelial cells, TNF-α- and lipopolysaccharide-mediated transcriptional activation of the human VCAM-1 promoter has been found to be mediated by NF-κB DNA elements, and the associated NF-κB DNA-binding protein is blocked by PDTC (35). PDTC has been shown to inhibit NF-κB stimulation by all inducers known including H₂O₂ (24), by preventing dissociation of the NF-κB/IκB complex (48). The antioxidative effect of thiol compound such as PDTC might hinge on a metal-chelating action as chelation of free iron, and copper is thought to be an important protective mechanism against oxidants. On the other hand, dithiocarbamates can act directly as free radical scavengers. In our experiments, the ability of the antioxidant PDTC to prevent the TNF-α-induced increase in VCAM-1 mRNA in SMCs supports an NF-κB-dependent signaling pathway for the cytokine-induced VCAM-1 expression. In contrast, the persistence of VCAM-1 mRNA expression after PDTC treatment of redifferentiated SMCs points to an NF-κB-independent pathway in this population of cells.

Taken together, these results indicate that VCAM-1 expression on SMCs is regulated by two independent pathways, an NF-κB-dependent pathway after cytokine activation and an NF-κB-independent pathway during the redifferentiation process.

Several mechanisms could account for the regulation of VCAM-1 expression during the redifferentiation process, either nonspecific or specific. Among the nonspecific mechanisms of VCAM-1 regulation during differentiation, an autocrine/paracrine activation comes to mind. However, the lack of involvement of NF-κB proteins in the transcriptional regulation suggests that VCAM-1 expression is independent of an autocrine/paracrine stimulation by growth factors such as PDGF-BB, bFGF, EGF, and IGF-1 or cytokines as TNF-α and interleukin-1α synthesized by arterial wall cells. All these secreted factors are known to activate the NF-κB system. Another possibility is the involvement of pleiotropic transcription factors, different from the NF-κB system. Two GATA DNA elements are present in opposite transcriptional orientations in the VCAM-1 gene promoter at positions between −255 and −235 relative to the transcription start site. GATA zinc finger proteins, which are involved in TNF-α induction of VCAM-1 in endothelial cells, belong to a transcription factor family composed of at least eight members. GATA-1, -2, -3 are involved in differentiation of hematopoietic cells. GATA-4 has a tissue distribution limited to heart and endothelially derived tissue. Furthermore, inhibition of GATA-4 protein expression blocks cardiac muscle differentiation in vitro. Recently, GATA-6 human transcripts have been detected in lung and liver and in cultures of human and rat vascular SMCs (49). In SMCs, GATA-6 transcripts are down-regulated when quiescent cultures are stimulated to proliferate in response to mitogen activation. As withdrawal from the cell cycle is a prerequisite for differentiation, it is possible that the GATA-6 protein participates in regulation of the differentiated state of SMCs and thus to induction of the VCAM-1 gene expression. However, a search for GATA DNA-binding proteins in redifferentiated SMC nuclear extracts using the GATA probe on EMSA did not uncover any specific DNA-protein complexes (data not shown).

VCAM-1 expression could be also regulated by a muscle-specific differentiation pathway. During striated muscle differentiation, VCAM-1 is expressed in a developmentally specific patter. The cell-cell interactions underlying myogenesis have been shown to involve an interaction between VCAM-1 and VLA-4 (4). Study of VCAM-1 promoter activity in C2C12 mouse myoblasts has identified a position-specific enhancer located between bp −17 and −5, consensus for an interferon regulatory factor (IRF) binding element that overrides the effect of the other promoter elements, resulting in VCAM-1 constitutive expression (50, 51). The transcription factor IRF-2 that interacts with this VCAM-1 gene promoter in muscle cells is expressed concomitantly with VCAM-1 in differentiating skeletal muscle in mouse embryos. Similarly, VCAM-1 expression has been described in human aorta during ontogenesis (8), suggesting that VCAM-1 could be regulated by this IRF binding site. However, in human differentiated SMCs, we did not find any specific protein-DNA complex by EMSA with this element (data not shown). These negative results suggested that IRF binding site is not involved in SMC expression of VCAM-1.

These results led us to suspect a smooth muscle-specific transcriptional regulation of VCAM-1 gene in differentiated SMCs. Several studies on smooth muscle gene promoters (SM22α, sm-MHC, sm-α actin) have found evidence for roles of various transcription factors such as MEF2 (52, 53) and CArG-box binding proteins (53, 54). Several potential CArG-boxes and MEF2 binding sites are present in the VCAM-1 gene promoter. The isofoms of the MEF2 transcription factor belonging to the MADS box family transcription factors are expressed in vascular SMCs and in vascular tissue (55) and during Drosophila ontogenesis, MEF2 is required for later aspects of differentiation of the three major types of musculature (56, 57). MEF2 binding sites are found in the control of the majority of muscle-specific genes (58). Interestingly, a MEF2-like binding site is present in the VCAM-1 gene promoter located from −929 to −982 (5′-TTTTAATAAA-3′). It resembles the MEF2-like sequence of the rabbit sm-MHC gene promoter found at −1540 to −1530 (5′-TATTAAATAAA-3′) involved in gene expression in rat aortic SMCs (52). It is of note that this MEF2-like binding site specifically interacts with MEF2B (59). These findings suggest that many potential smooth muscle-specific cis-regulator elements in the VCAM-1 gene promoter could participate in the regulation of VCAM-1 during redifferentiation of smooth muscle cells. This might constitute a smooth muscle-specific pathway. Further experiments will be required to gain further understanding of the mechanisms underlying the intriguing pattern of VCAM-1 expression in smooth muscle tissue.

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