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Authors: Muroya, Susumu, Nakajima, Ikuyo, and Chikuni, Koichi

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Bovine Skeletal Muscle Cells Predominantly Express a Vascular Cell Adhesion Molecule-1 Seven-Ig Domain Splice Form

Susumu Muroya*, Ikuyo Nakajima and Koichi Chikuni

Department of Animal Products, National Institute of Livestock and Grassland Science, Tsukuba, Ibaraki 305-0901, Japan

ABSTRACT—Vascular cell adhesion molecule-1 (VCAM-1), which has several alternatively splicing variants, plays a role in myotube formation. To investigate which form functions in myogenesis, we analyzed VCAM-1 mRNA expression in bovine skeletal muscle cells. We detected the expression of two VCAM-1 splice forms in the muscle tissue and in the primary satellite cell culture. The longer form was predominantly expressed at the muscle and during myotube formation of the cells. The nucleotide sequences of the two forms were determined by cDNA direct sequencing. The sequence data showed that the predominant form in skeletal muscle was a full-length VCAM-1 (VCAM-7D) that consists of seven immunoglobulin-like (Ig) domains, and the minor form was a novel six-domain form that lacks the seventh Ig domain. Compared to this, bovine pulmonary artery endothelial cells also express a variant which lacks domain 7, but VCAM-7D was not detected by RT-PCR in the culture. No VCAM-1 expression was detected in bovine kidney epithelial cell, lymph node epithelial cell, or leukemic B-lymphocyte culture even under stimulation by tumor necrosis factor-α. These data suggest that the splicing of the VCAM-1 gene alternatively varies depending on the cell type where it is expressed, and that VCAM-7D plays a predominant role in myotube formation.

INTRODUCTION

Vascular cell adhesion molecule-1 (VCAM-1) has been identified in activated human umbilical vein endothelial cells (HUVEC) by monoclonal antibody E1/6 (Rice and Bevilacqua, 1989), and by expression cloning (Osborn *et al*., 1989). VCAM-1 is not constitutively expressed and is induced on the cell surface in vitro by exposure to lipopolysaccharide (LPS) and inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Osborn *et al*., 1989; Rice and Bevilacqua, 1989). VCAM-1 mediates adhesion of melanoma (Rice and Bevilacqua, 1989) and leukocytes (Osborn *et al*., 1989) to endothelial cells via interaction with counter-receptor VLA-4, which is a heterodimer of integrin, subunits α4 and β1 (Elices *et al*., 1990).

VCAM-1 has also been known to play a role in myotube formation. In mammalian skeletal muscle differentiation (Ross *et al*., 1987; Stockdale and Miller, 1987; Ontel *et al*., 1988), an early-born population of myoblasts (primary myoblasts) fuse to form primary myotubes, after which a distinct population of myoblasts (secondary myoblasts) align along the primary myotubes. The primary myotubes apparently function as a template for secondary myotube formation by secondary myoblasts. Most of adult skeletal muscle fibers are composed of secondary myotubes. α4β1 integrin is present on primary myotubes, and VCAM-1 is present on secondary myoblasts and portions of secondary myotubes that are apposed to primary myotubes (Rosen *et al*., 1992; Sheppard *et al*., 1994; Jesse *et al*., 1998). These data suggest that these adhesion molecules play a role in the alignment of secondary myoblasts along primary myotubes and/or the subsequent fusion of the secondary myoblasts into secondary myotubes. Furthermore, in adult skeletal muscle, VCAM-1 expression persists on the satellite cells (Rosen *et al*., 1992; Jesse *et al*., 1998), which are wedged between the basal lamina and the muscle fibers. This finding suggests that α4β1 integrin and VCAM-1 may also have a role in muscle repair (Dean *et al*., 1993; Jesse *et al*., 1998).

VCAM-1 belongs to the immunoglobulin superfamily (IgSF) and its molecular structure is characterized by multiple C2-type immunoglobulin-like (Ig) domains (Chothia and Jones, 1997). As is common among members of the IgSF, each Ig domain is encoded by a single exon and the alternative splicing leads to the addition or deletion of Ig domains (Cybulsky *et al*., 1991b). Human VCAM-1 can consist of a 6-domain form (Osborn *et al*., 1989) and a predominant 7-domain form (Polte *et al*., 1990; Cybulsky *et al*., 1991a), both of which are expressed in activated HUVEC. Mouse VCAM-1 has a 7-do-

* Corresponding author: Tel. +81-298-38-8686;
FAX. +81-298-38-8683.
E-mail: muros@affrc.go.jp

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main form in the lymph node, lung, and spleen after lipopolysaccharide (LPS) injection (Araki *et al.*, 1993), as well as in the spleen, kidney, heart, and brain (Terry *et al.*, 1993), and in the fibroblasts and endothelial cells (Kumar *et al.*, 1994). Mouse VCAM-1 also has an inflammation-specific glycophosphatidylinositol (GPI)-anchored three-domain form in LPS-treated lungs and kidneys (Moy *et al.*, 1993), IL-1β-treated spleens, kidneys, and hearts (Terry *et al.*, 1993) and IL-1β-treated fibroblasts and endothelial cells (Kumar *et al.*, 1994). Furthermore, rat VCAM-1 7-domain forms appears in LPS-treated lungs (Williams *et al.*, 1992), the rabbit 7- and 8-domain form appear in venous endothelial cells (Cybulsky *et al.*, 1991b), and the porcine 5-domain form appears in activated venous endothelial cells (Tsung *et al.*, 1994).

This variety of splicing products has raised the possibility of cell- or tissue-specific distribution and function of each VCAM-1 splice form. In fact, expression of mouse GPI-anchored VCAM-1 three-domain forms can be differentially regulated in a cell type- or stimulation-specific manner (Kumar *et al.*, 1994). In addition, neural cell adhesion molecule (NCAM), a member of the IgSF, has a variety of tissue-specific forms including a muscle-specific form (Cunningham *et al.*, 1987; Dickson *et al.*, 1987).

Despite the presence of various VCAM-1 splice forms, the specific VCAM-1 splice form that functions in myotube formation has not yet been determined. In the present study, we investigated which VCAM-1 form is expressed in bovine muscle satellite cells, and examined the cell type specificity of splice form expression, using several types of bovine cells.

MATERIALS AND METHODS

Cell preparation and culture

To prepare bovine muscle satellite cells for our study, a biceps femoris muscle was excised within 3 min after slaughter from a six-month-aged Holstein cow bred in the National Institute of Animal Industry. The muscle was minced well and then was digested in a Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY) containing 0.1% collagenase (Sigma Chem., St. Louis, MO), 0.1% hyaluronidase (Wako Pure Chem., Osaka, Japan), 1000 U/ml dispase (Godo Shusei, Tokyo, Japan) at 37°C for 20 min. Digested tissue was filtrated by 70 μm mesh filter. After centrifugal purification, the obtained cells were cultured as primary skeletal muscle satellite cells in a DMEM containing 10% fetal calf serum (FCS) (Biological Industries, Kibbutz Beit Haemek, Israel) and 100 IU/ml penicillin (Sanko Pure Chem., Tokyo, Japan) and 100 mg/ml streptomycin (Sanko Pure Chem.). The cells were then maintained in a humidified atmosphere of 5% CO_2 at 37°C, and the medium was renewed every third day. To encourage the mytoube formation, the cells were induced to differentiate by serum-free COSMEDIUM (Cosmobio, Tokyo, Japan) when they reached confluence.

Bovine pulmonary artery endothelial (CPAE) cells, kidney epithelial (MDBK) cells, lymph node epithelial (23CLN) cells, and leukemic B-cell (KU-1) were purchased from RIKEN Cell Bank (Tsukuba, Japan). The CPAE, MDBK, and 23CLN cells were cultured in 20% FCS-containing minimum essential medium (MEM) (Sigma Chem.), 10% FCS-DMEM, and 10% FCS-MEM, respectively, under the same conditions as the muscle cell culture. To induce VCAM-1 expression in these cultures, recombinant human TNF-α (Gibco) was added at 25 ng/ml for 24 hrs into CPAE, MDBK, and 23CLN cell cultures that reached 1×10^4 cell/cm^2, and into KU-1 cell culture that reached 1.1×10^5 cell/ml.

mRNA preparation and nucleotide sequence determination

Using ISOGEN (NipponGene, Tokyo, Japan), total RNA was extracted from the bovine biceps femoris and the satellite cells at several culture points from the growth phase to the myotube formation phase, and from the other cells tested after the TNF-α treatment.

The first-strand cDNA was synthesized from 0.5 μg of total RNA using M-MLV Reverse Transcriptase RNase-H minus (Toyobo, Tokyo, Japan) with the primer 3ADP1. The sequences of all the primers used in this study are shown in Table 1. The muscle cells cultured for 4 days after induction of differentiation were used as the cDNA source for the VCAM-1 nucleotide sequence determination. Initially we used primers designed for human VCAM-1 cDNA sequence (Osborn *et al.*, 1989; Polte *et al.*, 1990; Cybulsky *et al.*, 1991a). Using the obtained cDNA as a template, we first amplified a fragment using a primer set, vcam-2 and vcam-R2. We attempted to extend the sequencing both upward and downward from the fragment with AmpliTaq Gold (Perkin-Elmer, Foster City, CA). After nucleotide sequence determination of this fragment, bovine specific vcR4 was designed in the fragment and was used with vcam-6 in order to determine the first upward fragment. To promote upward extension, primer sets vcam-10 and vcR5, and vcam-11 and vcR7 were used for the second and the third fragments, respectively.

To promote downward extension from the vcam-2-vcam-R2 fragment, vc4 and 3ADP1 were used for the next fragment amplification. This amplification generated two fragments with a difference in length of approximately 300 bp. For the longer fragment, we first determined the sequence and then designed a primer vc7 for this bovine fragment. A single fragment was amplified by using this primer and 3ADP1.

All bands of the amplified fragments were purified by excision from 4% agarose gel after electrophoresis. Using these fragments as templates, a second PCR and the subsequent purification were performed. These purified products were used as templates for direct

Table 1. Nucleotide sequences of PCR primers used in this study

For nucleotide sequence determination[a]	
Forward	
vcam-2	5'-GAAATGACCTTCATCCCTACCATTGAAGATAC-3'
vcam-6	5'-AGGGTCTACCAGCTCCAGAGATTTTCTG-3'
vcam-10	5'-AAGATGGTCGTGATCCTTGGAGCCTCA-3'
vcam-11	5'-GGCCTCACTGGCTTCAGGAGCTGAA-3'
vc4	5'-TGAGAATGCAACTCTCAGCTTCATGTCTAC-3'
vc7	5'-CTTACAGCTTTTCCTTCTGAGAGGGTCA-3'
Reverse	
vcam-R2	5'-CTTCTTCCAGCCTGGTTAATTCCTTCAC-3'
vcR4	5'-GTCTGGCTCGGTTACCAATTGATGAAATG-3'
vcR5	5'-CGACAGCTCCTTTCTGAGAATGCAAC-3'
vcR7	5'-AGCTTCCCAAATCGACATATTCCCAAGTGA-3'
3ADP1	5'-CTGCAGGAATTCGATATCGAAGCTTGC-3'
For splicing variety[b]	
Forward	
vc11	5'-CAGCTAAGTAATGGGGATCTACAGCCTA-3'
vc13	5'-ATGCTTAGGAAGATGTTTGTGATCTTTGGA-3'
vc15	5'-GATGATTCTCCACCCAAAGTAAGGAAAACT-3'
vc16	5'-CAACTCTCACTTTAATTTCTATGAGGGCAG-3'
Reverse	
vcR11	5'-GTAGAAGCACAGAAAGTCTGCTAATGCTTG-3'
vcR13	5'-ACTTTACTGTTGAGATCTCTCCTGGAC-3'
vcR15	5'-GCATATACCATCCACAGGGCTCAGTTA-3'

[a] vc4, vc7, vcR4, vcR5, and vcR7 were based on bovine nucleotide sequence, and the others were based on human sequence.
[b] All the primers were based on bovine nucleotide sequence.

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sequencing with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) and 373S DNA sequencer.

PCR primers and analysis

In order to determine the VCAM-1 splicing variety, specific primers for bovine VCAM-1 were designed (Table 1) and were used to detect spliced-out domains. In this experiment, the muscle cells cultured for 6 days after differentiation induction were adopted as the sample of PCR templates. The fragment between vc16-vcR15 was amplified using AmpliTaq Gold and the other fragments were amplified using LA-PCR Kit (Takara, Tokyo, Japan). The PCR procedures using the AmpliTaq Gold were carried out first for 9 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 60°C, and 1 min at 72°C, and finally 7 min at 72°C in the presence of 5% dimethylsulfoxide. Amplification using the LA-PCR Kit was performed for 1 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C, and 4 min at 72°C, and finally 10 min at 72°C. In order to determine whether or not domain 7 had been spliced out, a primer set vc11-vcR11 was also used with AmpliTaq Gold. In the analysis of VCAM-1 expression in bovine cell cultures, ribosomal protein L7 (RPL7) was adopted as a standard. The nucleotide sequences of forward and reverse PCR primers of RPL7, 5'-GCAGAACCCAAATTGGCGTTTGTCATCAG-3' and 5'- GAAGACAATTTGAAGGGCCACAGGAAGT-3', respectively, were designed based on mouse RPL7 sequence.

RESULTS

In a preliminary experiment, we obtained two fragments, of approximately 1 kb and 1.3 kb, by amplification between VCAM-1 domain 6 and the 3' untranslated (3'UTR) region (vc4-3ADP1). The partial nucleotide sequences of the two fragments between domain 6 and 3'UTR region showed a deletion of 267 bp in the short fragment. Comparison with full-length human VCAM-1 nucleotide sequence indicated that the 267-bp region present in the long cDNA fragment was VCAM-1 domain 7. The result suggests that two VCAM-1 forms were derived by alternative splicing.

We next determined the complete coding mRNA sequences of the two bovine VCAM-1 forms. The nucleotide sequences of the two forms revealed that the long form was full-length VCAM-1 which consists of seven Ig domains (VCAM-7D), and the short form was a variant lacking the seventh Ig domain (VCAM-6DΔ7) (Fig. 1). Bovine VCAM-7D and VCAM-6DΔ7 cDNA are predicted to encode for proteins of 739 and 650 amino acids (aa), respectively. By analogy to sequences of other species (Polte *et al.*, 1990; Cybulsky *et al.*, 1991a; Cybulsky *et al.*, 1991b; Williams *et al.*, 1992; Araki *et al.*, 1993; Tsung *et al.*, 1994), the region of the first 24 aa serves as a signal peptide (SP). The mature proteins of VCAM-7D and VCAM-6DΔ7 have 663- and 574-aa extracellular regions, respectively, with a 22-aa transmembrane (TM) region and an 18-aa cytoplasmic (CP) tail. In comparison with other mammals by overall amino acid sequence, more than 70% of the entire amino acid residues of VCAM-1 were conserved. Five potential N-glycosylation sites were found in the region of domains 3-6, three locations (asparagine residues at positions 274, 532, and 562) of which were conserved in each of all mammalian VCAM-1s previously reported. Cysteine residues of each domain, that are crucial to fold the functional Ig domain structures (Jones *et al.*, 1995; Wang *et al.*, 1995; Wang and Springer, 1998), were also completely conserved among mammalian species previously studied.

The amino acid sequence of each bovine VCAM-1 Ig domain is highly similar to that of other mammals (Table 2). Domains 1 and 4 are independently essential for the VCAM-1 molecule to bind with α4 integrin (Osborn *et al.*, 1992; Vonderheide *et al.*, 1992; Vonderheide *et al.*, 1994). The key α4 integrin-binding motif, IDSPL was completely conserved in domains 1 and 4 of bovine VCAM-1 (Vonderheide *et al.*, 1994; Wang and Springer, 1998). Domain 7 was the most highly conserved among all of the Ig domains. In addition, internal similarity among Ig domains was found in bovine VCAM-1 as in the other mammalian sequences. In the case of bovine VCAM-1, the identities between domains 1 and 4, between domains 2 and 5, and between domains 3 and 6 were 55.9, 50.5, and 61.8%, respectively (Table 3). Among the other Ig domains, no identity of more than 35% was observed.

The nucleotide sequence data reported in this paper appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB052746 (VCAM-7D) and AB052747 (VCAM-6DΔ7).

To investigate the possible expression of the other VCAM-1 splice forms, we performed LA-PCR using primers designed for the complete coding region between the SP and CP regions (vc13-vcR11; 2227 bp) (Fig. 2A). Two fragments were amplified from cDNA templates of the bovine skeletal muscle satellite cell culture; their lengths were approximately 2.2 kb and 1.9 kb (Fig. 2B, SP-CP). These lengths correspond to the coding region length of the full-length form (VCAM-7D) and its variant with a 0.3-kb deletion, respectively. We next used various forward and reverse primer combinations (Fig. 2A) to examine whether or not domains other than domain 7 are spliced out. When a primer set designed for the region between SP and domain 4 (vc13-vcR13; 964 bp), or for the region between domains 3 and 7 (vc16-vcR15; 1159 bp) was used, only one fragment was observed (Fig. 2B, SP-D4 and D3-D7). The length of the SP-D4 fragment (approximately 1 kb) in Fig. 2B corresponds to that of the fragment containing no deletion in the SP-domain 4 region, while the length of the D3-D7 fragment (approximately 1.2 kb) corresponds to that of the fragment containing no deletion in the region of domains 3–7. This result suggests that the deleted domain was not present in the region of domains 1–6. On the other hand, two fragments were amplified by a primer set for the region between domain 2 and the CP region (vc15-vcR11; 1614 bp) (Fig. 2B, D2-CP). The upper (approximately 1.6 kb) and lower (approximately 1.3 kb) fragments of D2-CP in Fig. 2B were expected to be a full-length fragment and a 0.3 kb-deleted fragment, respectively, of the domain 2-CP region. These results indicate that one of domains 3, 4, 5, 6, or 7 was absent in the short variant. Taken together, these data suggest that bovine muscle satellite cells express only two VCAM-1 splice forms: full-length VCAM-7D and the novel variant VCAM-6DΔ7. No other splice variant of VCAM-1 was detected in the muscle cell culture in this study.

To examine the expression pattern of the two VCAM-1

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      -77  GACCCTTCCCAGGCACCTTCAGGAGGGACAGAAAGAAGCATTTTGGAATAATTTTCTCTT
      -17  AAGAGCAGCAACTTAAAATGCTTAGGAAGATGTTTGTGATCTTTGGAGCCTCAGATGTAC
                             M  L  R  K  M  F  V  I  F  G  A  S  D  V      14
       44  TTTGGATGGTGTTTGCAGTTTCTCAAGCTT
           L  W  M  V  F  A  V  S  Q  A
D1                                     CCCAAATCGACATATTCCCAAGTGAATCCA
                                       S  Q  I  D  I  F  P  S  E  S       34
      104  AAATTTTTGCGCAGATTGGTGACTCTGTTTCACTGACTTGCAGCACAACAGGCTGTGAGT
           K  I  F  A  Q  I  G  D  S  V  S  L  T  C  S  T  T  G  C  E     54
      164  CCCCATCATTGTCTTGGAGAACTCAGATTGACAGTCCACTGAATGGAAAGGTGAAAACTG
           S  P  S  L  S  W  R  T  Q [I  D  S  P  L] N  G  K  V  K  T     74
      224  AGGGGACCACATCCATGCTGATCATGGATCCTGTTAGTTTCAAGGATGAACACCAGTACC
           E  G  T  T  S  M  L  I  M  D  P  V  S  F  K  D  E  H  Q  Y     94
      284  TGTGCACAGCGATTTGCAAGGATAAGAAACTGGAGAAAGCAATCCGAGTGGAGATCTACT
           L  C  T  A  I  C  K  D  K  K  L  E  K  A  I  R  V  E  I  Y    114

D2    344  CTTTCTCTAAGGATCCAGAGATTCATTTGAGTAGCCCCCCAGAGGTCGGGAAGCCAGTCA
           S  F  S  K  D  P  E  I  H  L  S  S  P  P  E  V  G  K  P  V    134
      404  CAGTCACATGTTCGGTGCCTGATGTTTACCCATTTGAAAGGCTGGAGATAGAGTTGTTGA
           T  V  T  C  S  V  P  D  V  Y  P  F  E  R  L  E  I  E  L  L    154
      464  AAGGCAACCGTCCCATGAAAGTACAGGACTTTCTGGAGCCTTCAGAAAAAAAGGCCCAGG
           K  G  N  R  P  M  K  V  Q  D  F  L  E  P  S  E  K  K  A  Q    174
      524  AAACAAAGAGCTTGGACGTGACCTTCTCGCCTACTGATGAGGATATTGGGACAGCTCTTG
           E  T  K  S  L  D  V  T  F  S  P  T  D  E  D  I  G  T  A  L    194
      584  TTTGTCAAGCTACATTACACATTTATGATGATGATTCTCCACCCAAAGTAAGGAAAACTA
           V  C  Q  A  T  L  H  I  Y  D  D  D  S  P  P  K  V  R  K  T    214
      644  CAAAAGAACTGGAAGTCTACA
           T  K  E  L  E  V  Y
D3                                 TCTCCCCCAAAGACACGGCTATATCCGTGAATCCCTCCA
                                   I  S  P  K  D  T  A  I  S  V  N  P  S    234
      704  CAAGGCTGCAGGAAGGCGACTCCGTGACGATGACGTGTGCCAGTGCAGGTTTACCAGCTC
           T  R  L  Q  E  G  D  S  V  T  M  T  C  A  S  A  G  L  P  A    254
      764  CACGGATTCTCTGGAGCAAGAAATTAGACAATGGGAACCGACAGCTCCTTTCTGAGAATG
           P  R  I  L  W  S  K  K  L  D  N  G  N  R  Q  L  L  S  E  N    274
      824  CAACTCTCACTTTAATTTCTATGAGGGCAGAAGATTCTGGGATTTATGTGTGTGAAGGAA
           A  T  L  T  L  I  S  M  R  A  E  D  S  G  I  Y  V  C  E  G    294
      884  ATAACCCTGTTGGGAAAGACAGAAAAGAGGTGAAATTAACTGTTCAAG
           N  N  P  V  G  K  D  R  K  E  V  K  L  T  V  Q
D4                                                             AGAAAAACTTTA
                                                               E  K  N  F    314
      944  CTGTTGAGATCTCTCCTGGACCCCAGATTGCTGCTCAGGTTGGCGACTCAGTCGTGTTGA
           T  V  E  I  S  P  G  P  Q  I  A  A  Q  V  G  D  S  V  V  L    334
     1004  CCTGTGATGTCAGGGACTGCGAGTCCCCATCTTTCTCTTGGAGAACACTGATAGACAGCC
           T  C  D  V  R  D  C  E  S  P  S  F  S  W  R  T  L [I  D  S    354
     1064  CTCTGAATGGGAATGTGAGGAGTGAGGGCTCCAAGTCCACGTTGACCCTGAGCCCAGTGA
           P  L] N  G  N  V  R  S  E  G  S  K  S  T  L  T  L  S  P  V    374
     1124  GTTTTGAGAACGAACATTTCTACCTGTGCACTGTGATGTGTGGGCAAAAGAAACTGGAAA
           S  F  E  N  E  H  F  Y  L  C  T  V  M  C  G  Q  K  K  L  E    394
     1184  AGAGAATCCAGGTGAAGCCCTACT
           K  R  I  Q  V  K  P  Y
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D5                                      CCTTCCCTAGCAATCCAGAAATTGAAATGAGTGGTC
                                        S  F  P  S  N  P  E  I  E  M  S  G    414
1244  CATTAGTGAGTGGAAACCCAGTCACTGTAAGCTGCAGGGTTCCTAATGTGTATCCTTCTG
      P  L  V  S  G  N  P  V  T  V  S  C  R  V  P  N  V  Y  P  S    434
1304  ACCAGCTGGAGATTGAATTGTTTAAAGGGGAGAGTATTATGATGAATAAAACCTTCTCGA
      D  Q  L  E  I  E  L  F  K  G  E  S  I  M  M  N  K  T  F  S    454
1364  AAGATGTAAGTAAGAAAGCCCTAGAGACCAAGAGTTTGGAGAAGACCTTCATCCCCACCA
      K  D  V  S  K  K  A  L  E  T  K  S  L  E  K  T  F  I  P  T    474
1424  CTGAAGATATTGGAAATGTTCTTGTTTGTCTGGCTCGGTTACCAATTGATGAAATGGAAT
      T  E  D  I  G  N  V  L  V  C  L  A  R  L  P  I  D  E  M  E    494
1484  TTGAACTCAAACAAAGGCAGAGTACACAAACACTTTATGTTAATG
      F  E  L  K  Q  R  Q  S  T  Q  T  L  Y  V  N
D6                                                     TTGCTCCCAGGGATA
                                                       V  A  P  R  D    514
1544  CAACCATCTTGGTCAGTCCCTCCTCCATCCTGGAGGAAGGTAGTTCTGTGAATATGACAT
      T  T  I  L  V  S  P  S  S  I  L  E  E  G  S  S  V  N  M  T    534
1604  GCTCTAGCAATGGCCTTCCAGCTCCCAAAATCTTGTGGAGCAGGCAGCTAAGTAATGGGG
      C  S  S  N  G  L  P  A  P  K  I  L  W  S  R  Q  L  S  N  G    554
1664  ATCTACAGCCTATTTCTGAGAATGCAACTCTCAGCTTCATGTCTACAAAAATGGAAGATT
      D  L  Q  P  I  S  E  N  A  T  L  S  F  M  S  T  K  M  E  D    574
1724  CTGGTATTTATGTCTGTGAAGGCATTAACCAGGTTGGAACAAGCAGAAAAGAAGTCAACT
      S  G  I  Y  V  C  E  G  I  N  Q  V  G  T  S  R  K  E  V  N    594
1784  TAATTATCCAAG
      L  I  I  Q
D7                    TTGCTCCAAAAGATATACAACTTACAGCTTTTCCTTCTGAGAGGGTCA
                      V  A  P  K  D  I  Q  L  T  A  F  P  S  E  R  V    614
1844  AGGAAGGAGACACTGTCATTATCTCCTGTACGTGTGGAAATGTTCCCCCAACTTTGATAA
      K  E  G  D  T  V  I  I  S  C  T  C  G  N  V  P  P  T  L  I    634
1904  TTCTGAAGAAAAAAGCAGAGACTGGCTACACAGTGCTAAAGTCTACAGGTGGTGCATATA
      I  L  K  K  K  A  E  T  G  Y  T  V  L  K  S  T  G  G  A  Y    654
1964  CCATCCACAGGGCTCAGTTAGAGGATGCGGGAGTATACCAGTGTGAATCTAAAAATGAAG
      T  I  H  R  A  Q  L  E  D  A  G  V  Y  Q  C  E  S  K  N  E    674
2024  TTGGCTCACAGCTAAGAAGCCTAACACTTGATGTTAAAG
      V  G  S  Q  L  R  S  L  T  L  D  V  K
                                            GAAGAGAAAGTAACAAGGACT
                                            G  R  E  S  N  K  D    694
2084  ATTTTTCTCCTGAACTTCTGGTGCTCTATTGTGCATCCTCCTTAATAATACCTGCCATTG
      Y  F  S  P  E  L  L  V  L  Y  C  A  S  S  L  I  I  P  A  I    714
2144  GAATGATTATTTACTTTGCTAGAAGAGCCAACATGACAGGGTCATACAGTCTTGTAGAAG
      G  M  I  I  Y  F  A  R  R  A  N  M  T  G  S  Y  S  L  V  E    734
2204  CACAGAAAGTCTGCTAATGCTTGTAATACAAGTGTAGCTACTGCGTGAAAGGTTCTACCA
      A  Q  K  V  C  *                                              739
2264  AAGATACTATCAGTTCAGATGCTCAATACTGCTCATCATTCCACAAGGAAAACAGAAACT
2324  GGGGGTTCAGGCTTCCTTGAATGCAGTGAAGGCTTGGAAAGAAATAGCTGCCTATGGCCC
2384  TTACTGTGAGCAAGAAGCCAAAGGAAAATTTCTACTTAAAAACTAGGCACTACCACTGAG
2444  ATGACTGGAGTAATTTCTTGATGTGTACATACAATAACATGATTTGTATATATGTAAAAC
2504  TATGCTGTAGCAAGGTTGTTTGGAATATCA
```

Fig. 1. Nucleotide and deduced amino acid sequence of bovine VCAM-1 cDNA. Signal peptide, transmembrane region, and cytoplasmic region are on wavy, dashed, and single underlines, respectively. Ig domain 7 is double underlined. Each Ig domain is marked with the number from D1 to D7. Closed diamonds and open triangles indicate the cysteine residues that are predicted to form disulphide bridge, and potential N-glycosylation sites, respectively. Boxed IDSPL motifs are α4 integrin-binding sites.

forms in the muscle cell culture by detection of the domain 6-CP region, multiplex PCR was carried out with an internal standard ribosomal protein L7 (RPL7) using a primer set vc11 and vcR11. Myotubes were observed from day 15 in culture and, by the last stage in culture (day 18), multinucleated myotubes were frequently formed (Fig. 3B). In the cells in the growth phase (Fig. 3A) and during the myotube formation (Fig. 3B) as well as the tissue, the 579-bp fragment corresponding to the full-length fragment of the domain 6-CP region, and the 312-bp fragment corresponding to the domain 6-CP region

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Table 2. Comparison of the domains of bovine and the other mammalian VCAM-1

Domain	Amino acid identity to bovine VCAM-1 (%)[a]			
	human	mouse	rat	pig
Signal peptide	62.5	62.5	70.8	70.8
Domain 1	72.2	66.7	71.1	63.7
Domain 2	65.4	59.8	61.7	65.7
Domain 3	78.7	69.7	75.3	80.9
Domain 4	80.4	69.6	71.7	–
Domain 5	69.2	66.4	67.3	–
Domain 6	83.2	77.5	73.0	76.4
Domain 7	84.3	79.8	76.4	86.5
TM/CP	86.8	81.1	81.1	84.9
Overall	76.1	70.1	71.4	75.2

TM: transmembrane region, CP: cytoplasmic region.

[a] The percentage of amino acid identity has been calculated by counting the number of amino acids that are identical to each animal VCAM-1 and does not take into account nucleotide insertions or deletions.

Table 3. Intramolecular comparison of the Ig domains of bovine VCAM-1

	Amino acid identity to the other intramolecular Ig domain (%)[a]					
Domain	1	2	3	4	5	6
1	–					
2	19.6	–				
3	26.1	28.2	–			
4	55.9	22.0	27.2	–		
5	18.8	50.5	23.6	16.8	–	
6	24.7	24.3	61.8	20.4	18.7	–
7	19.0	19.6	33.7	15.8	23.9	27.0

[a] The percentage of amino acid identity has been calculated by counting the number of amino acids that are identical to each Ig domain of bovine VCAM-1 and does not take into account nucleotide insertions or deletions. Underlined numerals indicate over 50% identity.

Fig. 2. Alternative splicing analysis of VCAM-1 form variety by RT-PCR. (A) Location of primers used for splice form analysis. Ig domains are designated according to the number from D1 to D7. SP: signal peptide, TM: transmembrane region, CP: cytoplasmic region. (B) PCR results of various VCAM-1 regions. Primer sets used in this experiment are as follows: vc13-vcR11 for the SP-CP region, vc13-vcR13 for the SP-D4 region, vc15-vcR11 for the D2-CP region, and vc16-vcR15 for the D3-D7 region. DNA size marker is shown as kb on the left side.

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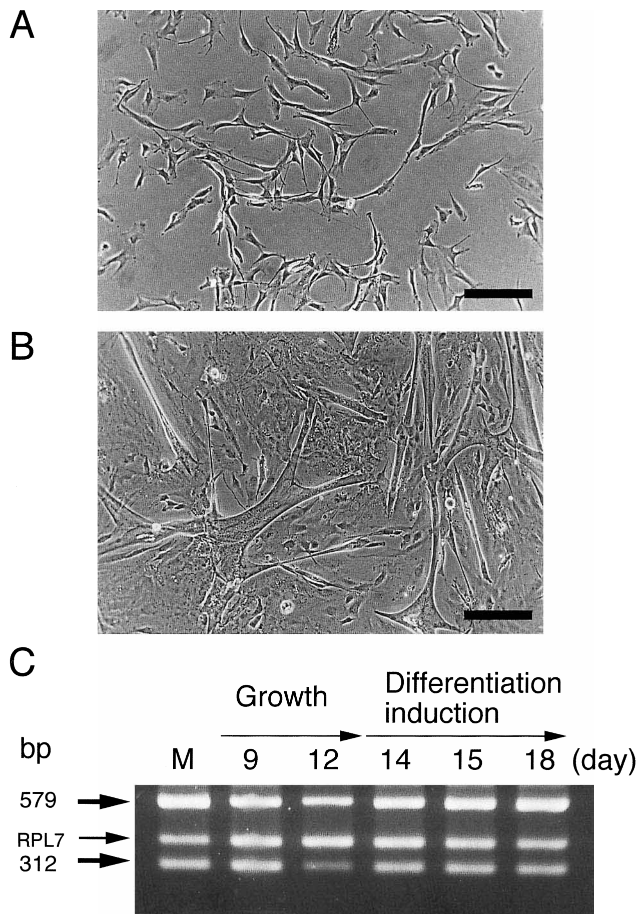

Fig. 3. Expression pattern of two VCAM-1 splice forms during myotube formation in bovine skeletal muscle satellite cells. (A) Satellite cells at growth phase (day 12). (B) Multinucleated myotubes formed by day 16. (C) Expression analysis of two VCAM-1 forms by RT-PCR. cDNA templates were synthesized from equivalent total RNAs of skeletal muscle tissue (M) and the cultured satellite cells at each culture point (from 9 d to 18 d) and were equivalently applied to the PCR. DNA size maker is given as bp on the left side. The bar in (A) and (B) indicates 200 μm. RPL7: ribosomal protein L7.

without domain 7 were observed (Fig. 3C), suggesting the expression of VCAM-7D and VCAM-6DΔ7 both at the muscle tissue and in the cultured cells. Through the entire culture duration including the growth and the myotube formation stage, VCAM-7D was predominantly expressed. By day 12, the expression of the two forms appeared to gradually increase (Fig. 3C), though how the gene is up-regulated remains unknown.

In order to investigate whether these forms are specifically expressed in muscle cells, we examined VCAM-1 expressions in the other bovine cells by amplifying the region of domain 6-CP (vc11-vcR11). The full-length fragment of the domain 6-CP region (579 bp) was predominantly observed in the skeletal muscle tissue and the primary cell culture (Fig. 3), however the fragment could not be detected in the pulmonary artery endothelial (CPAE) cell culture (Fig. 4). The fragment of the domain 6-CP region without domain 7 (312 bp) was observed in both the muscle and the TNF-α-treated the artery endothelial cell cultures. These observations reveal that

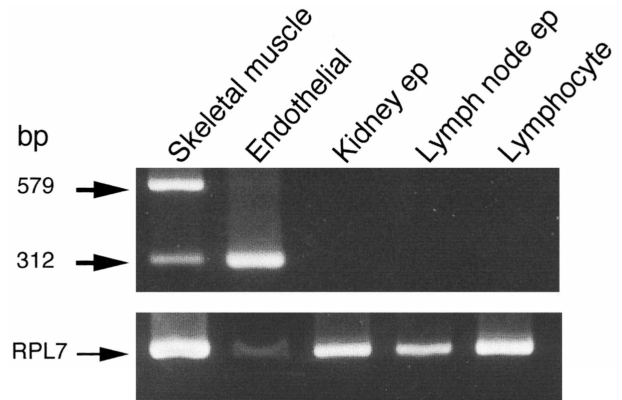

Fig. 4. Expression of VCAM-1 domain 7-containing form in various bovine cells. RT-PCR using a primer set vc11-vcR11 was performed for amplification of the domain 6-cytoplasmic region of VCAM-1 expressed in various bovine cells: skeletal muscle satellite cells (Skeletal muscle), CPAE cells (Endothelial), MDBK cells (Kidney ep), 23CLN cells (Lymph node ep), and KU-1 cells (Lymphocyte). Equivalent volume of each template synthesized from equivalent total RNA was applied to the PCR. DNA size marker is given as bp on the left side. RPL7: ribosomal protein L7.

primary muscle satellite cell cultures predominantly express VCAM-7D, while the artery endothelial cells express only a variant which lacks domain 7. No expression of any VCAM-1 spliced form could be detected in kidney epithelial cell (MDBK), lymph node epithelial cell (23CLN), or leukemic B-cell (KU-1) culture under TNF-α treatment by amplification of the domain 6-CP (Fig. 4) and the SP-CP regions (data not shown).

DISCUSSION

The amino acid sequences deduced from the nucleotide sequences of VCAM-7D and VCAM-6DΔ7 show the common structural features between the two forms. The domains 1 and 4, highly homologous to each other, have crucial α4 integrin-binding sites and therefore can function as the key domain of VCAM-1 (Osborn *et al*., 1992; Vonderheide *et al*., 1992; Vonderheide *et al*., 1994). Since the two VCAM-1 forms have the two key domains, both molecules can contribute to myotube formation through interaction with α4 integrin. The expressions of both functionally bivalent forms are consistent with a previous study which suggests the interaction of VLA-4 and VCAM-1 during myotube formation (Rosen *et al*., 1992). The amino acid sequences of domains 2, 3, and 5 that support the VCAM-1 molecular conformation and the binding function (Webb *et al*., 1993; Wang and Springer, 1998) are also well conserved. The amino acid sequences of domains 1, 2, and 3 are similar to those of domains 4, 5, and 6, respectively, which suggests an intergenic duplication in the evolutionary history of the VCAM-1 gene (Osborn *et al*., 1989; Wang and Springer, 1998).

Among these VCAM-1 Ig domains, the amino acid sequence of domain 7 is unique (Araki *et al*., 1993); the identities observed between domain 7 and the other domains were less than 33.5% in the bovine VCAM-1. Nevertheless, among

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all Ig domains, the sequence of this domain is the most highly conserved in human, mouse, rat, and pig VCAM-1 (Table 2). The presence of domain 7, the most C-terminal domain next to the TM region, is the structural difference between the VCAM-7D and the VCAM-6DΔ7. As for the role of the more C-terminal Ig domains, proteolysis experiments suggest that these domains have unusual structural features, which may be relevant to VCAM-1 function. The proteolytic sensitivity of domain 4, one of the two highly proteolysis-sensitive domains, is lost after domain 5, the other proteolysis-sensitive domain, is cleaved (Pepinsky *et al.*, 1992). These data suggest that the conformation of intact VCAM-1 molecules is strongly dependent on their C-terminal half, as well as on domain 3 (Webb *et al.*, 1993). Likewise, splicing of domain 7 may generate a conformational difference between VCAM-7D and VCAM-6DΔ7, which may be utilized by VCAM-1-expressing cells to modulate their adhesion ability.

The present RT-PCR results in detecting the splicing of domain 7 reveal that expressions of VCAM-7D and the novel variant VCAM-6DΔ7 appear to increase slightly in bovine muscle satellite cells, and that VCAM-7D is predominant from the growth phase to myotube formation. Expressions of both forms were also confirmed at each point during muscle cell differentiation by the amplification of the VCAM-1 entire coding region (SP-CP) with LA-PCR (data not shown). The expression pattern of both forms does not contradict that of previous studies in which VCAM-1 is demonstrated to be present on secondary myoblasts and portions of secondary myotubes (Rosen *et al.*, 1992; Sheppard *et al.*, 1994; Jesse *et al.*, 1998). A monoclonal antibody against VCAM-1 which blocks its interaction with α4β1 integrin inhibits fusion of mouse C2C12 cells (a satellite cell line) (Rosen *et al.*, 1992), which provides further evidence in favor of a role for VCAM-1 protein in myotube formation. Taken together, the predominant expression of VCAM-7D in skeletal muscle cells suggests that VCAM-7D plays an important role in myotube formation. Since the cells used in this study were primarily prepared, a role of VCAM-7D on the satellite cells in skeletal muscle repair was also suggested, as supported by previous reports (Dean *et al.*, 1993; Jesse *et al.*, 1998).

The minor form VCAM-6DΔ7 could be derived from the small number of fibroblasts and endothelial cells that are contained in the muscle tissue and the cell culture. However, we confirmed that more than 95% of the primarily cultured skeletal muscle cells were stained by anti-chicken desmin monoclonal antibody (Chemicon, Temecula, CA), which specifically reacts with bovine desmin in the cultured muscle cells (data not shown). Furthermore, the typical paving stone-shaped endothelial cells were rarely observed. Therefore, although the possibility of the effects of non-muscle cells on VCAM-6DΔ7 expression cannot be definitively ruled out, we conclude that bovine muscle satellite cells express a small amount of VCAM-6DΔ7 and predominantly express VCAM-7D. The cells expressing multiple VCAM-1 forms, such as the bovine muscle cells and HUVEC (Cybulsky *et al.*, 1991a), may control the ratio of the expressions of VCAM-1 splice variants and may regulate the adhesion ability.

We have shown that the two splicing products of a VCAM-1 gene were separately expressed depending on the cell type. The tissue- or cell-specific regulation of VCAM-1 alternative splice form expression has been predicted so far (Araki *et al.*, 1993; Terry *et al.*, 1993), because of the presence of various forms of VCAM-1 among species. Kumar *et al.* (1994) show that there can be differential regulation of the relative expression between the full-length form and the GPI-anchored three-domain form in response to cytokines, among rat smooth muscle cells, mouse fibroblasts and endothelial cells. In this study, bovine skeletal muscle satellite cells predominantly expressed the seven-Ig domain form VCAM-7D through the entire culture duration, including during the myotube formation stage. In comparison, pulmonary artery endothelial cell cultures exclusively express a variant lacking domain 7, which may be VCAM-6DΔ7. No other splice form, including the GPI-anchored three-domain form, could be detected in the muscle cell culture. In addition, no expression of any VCAM-1 splice form could be detected in kidney or lymph node epithelial cell culture. It is possible that there may be a different response in expression and splicing of VCAM-1 against TNF-α among the cells tested. However, Iademarco *et al.* (1993) demonstrated that VCAM-1 gene expression and the detected transcripts in mouse muscle cell line C2C12 was not affected by TNF-α. In addition, according to our RT-PCR analysis, only a variant lacking domain 7 was expressed at a basal level in the endothelial cell culture without TNF-α treatment (data not shown), suggesting no effect of TNF-α on the splicing of domain 7. These results suggest that alternative splicing of VCAM-1 mRNA is regulated in a cell type-specific manner, similar to that of NCAM (Cunningham *et al.*, 1987; Dickson *et al.*, 1987), although the regulation may vary among species. Whether domain 7 is necessary or not may vary according to cell type and cell-cell interaction. Furthermore, it is possible that, among various VCAM-1 splice forms, the bovine VCAM-7D plays a predominant role in myotube formation, and that its expression is largely, if not exclusively, confined to skeletal muscle.

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