Role of Transmembrane 4 Superfamily (TM4SF) Proteins CD9 and CD81 in Muscle Cell Fusion and Myotube Maintenance

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Abstract. The role of transmembrane 4 superfamily (TM4SF) proteins during muscle cell fusion has not been investigated previously. Here we show that the appearance of TM4SF protein, CD9, and the formation of CD9–β1 integrin complexes were both regulated in coordination with murine C2C12 myoblast cell differentiation. Also, anti-CD9 and anti-CD81 monoclonal antibodies substantially inhibited and delayed conversion of C2C12 cells to elongated myotubes, without affecting muscle-specific protein expression. Studies of the human myoblast-derived RD sarcoma cell line further demonstrated that TM4SF proteins have a role during muscle cell fusion. Ectopic expression of CD9 caused a four- to eightfold increase in RD cell syncytia formation, whereas anti-CD9 and anti-CD81 antibodies markedly delayed RD syncytia formation. Finally, anti-CD9 and anti-CD81 monoclonal antibodies triggered apoptotic degeneration of C2C12 cell myotubes after they were formed. In summary, TM4SF proteins such as CD9 and CD81 appear to promote muscle cell fusion and support myotube maintenance.

Key words: TM4SF proteins • CD9 • CD81 • myoblast • myotube

PROTEINS in the transmembrane 4 superfamily (TM4SF),[1] including CD9, CD37, CD53, CD63, CD81, CD82, and CD151, have been functionally implicated in cell proliferation, activation, motility, and tumor cell metastasis. In the plasma membrane, TM4SF proteins associate with each other and with other cell surface molecules, including CD4, CD8, CD19, CD21, major histocompatibility complex class I and II proteins, and integrins (26, 30, 40, 73). In addition, some TM4SF proteins may associate with cytoplasmic signaling molecules including tyrosine phosphatase (12), phosphatidylinositol 4-kinase (7, 78), and protein kinase C (PKC) (Zhang, X., and M.E. Hemler, manuscript submitted for publication). Through TM4SF proteins, these intracellular signaling molecules may be linked to the extracellular domains of integrins (24).

The CD81 protein may be a receptor for hepatitis C virus (54). Also, several TM4SF proteins may participate in virus-induced cell fusion. Antibodies against CD81 and CD82 inhibited human T cell leukemia virus type 1 (HTLV-1) but not HIV-1–induced syncytia formation (20). Overexpression of CD9 enhanced the susceptibility of cells to feline immunodeficiency virus (FIV) and canine distemper virus (39, 72), leading to elevated syncytia formation. Antibodies against CD9 reduced syncytia formation and/or virus production (17, 39, 72). Because the CD9 protein does not appear to directly bind to virus (39), it may act as a membrane fusion cofactor, rather than a primary virus receptor. Also, CD9 may play a role in the generation of multinucleate osteoclasts (64). From these results, we hypothesize that TM4SF proteins may play a general role in cell fusion events.

To extend this hypothesis, here we investigate the roles played by TM4SF proteins CD9 and CD81 in myogenic cell fusion. Integrins have been suggested previously to play a role during myogenesis (47, 57), as have other cell surface proteins including vascular cell adhesion molecule (VCAM)-1 (57), neural cell adhesion molecule (N-CAM) (18, 35), N-cadherin (36, 45), M-cadherin (79), meltrin (74), proteoglycans (56), and CDO (a protein with five Ig and three FNIII repeats) (34). However, a role for TM4SF proteins has not been demonstrated previously, and in fact, there have been few if any studies of TM4SF proteins on myoblasts or during myogenesis.

Here we have used the murine myoblast line C2C12 and the human rhabdomyosarcoma cell line RD to analyze the role of CD9 and CD81 during muscle cell fusion. CD9 and

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1. *Abbreviations used in this paper:* MFI, mean fluorescence intensity; MHC, myosin heavy chain; N-CAM, neural cell adhesion molecule; PKC, protein kinase C; TM4SF, transmembrane 4 superfamily; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling.

CD81 were chosen for study because they are both prominently expressed on skeletal muscle (50, 58) and on murine myoblast C2C12 cells (see below), and because mAbs to murine CD9 and CD81 are available. Our results suggest that CD9 and CD81 may regulate fusion of both C2C12 and RD cells, and may also regulate myotube maintenance.

Materials and Methods

Cell Lines

The human rhabdomyosarcoma line RD was obtained from American Type Cell Collection. RD cells were transfected with integrin α3 cDNA to yield RD-A3 as described previously (71). RD-C9-1 and RD-C9-2 are stable, unsorted lines independently derived by transfecting RD cells with full-length CD9 cDNA (generated by reverse transcriptase PCR) that was cloned into the expression plasmid pZeoSV (Invitrogen). Likewise, RD-A3C9-1 and RD-A3C9-2 are independently prepared stable transfectants derived from RD-A3 cells. All RD cells and transfectants were cultured in DME supplemented with 10% FCS. To induce differentiation and syncytia formation, subconfluent RD cells were cultured in 2% FCS. The mouse myogenic cell line C2C12 (8) was obtained from American Type Cell Collection and maintained in DME supplemented with 20% FCS (growth medium). To induce differentiation of confluent C2C12 cells, growth medium was replaced with DME containing 2% horse serum (differentiation medium) (57). To avoid loss of fusion competence, C2C12 cells were passaged only a few times before use.

Antibodies

Rat anti–mouse mAbs KMC8, anti-CD9; R1-2, antiintegrin α4; MFR5, antiintegrin α5; KMI6, antiintegrin β1; KM114, anti-CD44; R35-95, rat IgG2a negative control antibody; and hamster anti–mouse mAbs, HMβ1-1 and Ha2/5, antiintegrin β1, were all obtained from PharMingen. Hamster anti–mouse CD81 mAb 2F7 was purchased from Southern Biotechnology. Rat anti–mouse mAb CY8.2, antiintegrin α7, was a gift from Dr. Randall Kramer, University of California, San Francisco, San Francisco, CA. Rabbit polyclonal antibody against the integrin α3A cytoplasmic tail was provided by Dr. J.A. McDonald (Mayo Clinic, Scottsdale, AZ). Mouse anti–human N-CAM mAb, NCAM-OB11; anti–N-cadherin mAb, GC-4; and anti-pan cadherin cytoplasmic tail mAb, CH-19, all of which cross-react with mouse, were purchased from Sigma Chemical Co. Mouse mAbs MY-32, anti–myosin heavy chain (MHC); and DE-U-10, antidesmin, were also obtained from Sigma Chemical Co., and mouse antiactin mAb, C4, was purchased from ICN Biomedicals, Inc. Mouse anti–human mAbs were against integrin α5, A5-PUJ2 (55); CD9, BU16 (Biodesign International), DU-ALL-1 (Sigma Chemical Co.), C9-BB (6); and CD81, M38 (20).

Assay for C2C12 Myotube Formation

C2C12 cells were plated onto a 96-well tissue culture plate at 20,000 cells/well and cultured in growth medium. At confluence, growth medium was replaced by differentiation medium, in the presence of various mAbs. Numbers of myotubes in four independent 2.6-mm^2 fields were scored by microscopy as described (68). Elongated, glossy myotubes were easily distinguished from unfused myoblasts (see Fig. 4). In some experiments, numbers of myotubes per well that were longer than 250 μm were determined using the program Scion Image 1.60 (Scion Corp.), to acquire and analyze computer images from an Axiovert 135 microscope (Carl Zeiss) (as described in detail elsewhere; Stipp, C.S., and M.E. Hemler, manuscript submitted for publication).

Apoptosis Analysis

C2C12 cells were plated onto an 8-well chamber Permanox slide (Nunc) and precoated with mouse laminin (GIBCO BRL). Confluent cells were cultured in differentiation medium (with or without anti-TM4SF antibodies) for 6 d and then fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100, 0.1% sodium citrate. Nuclei of apoptotic cells were stained by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) (21), using In Situ Cell Death Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions. Immunofluorescence was analyzed using an Axioscop (Carl Zeiss).

For DNA ladder analyses, C2C12 cells were cultured for 6 d in differentiation medium, and then myotubes were detached from mixed myoblast/myotube cultures by treatment with 0.01% trypsin, 0.004% EDTA for 15 min as described previously (5). DNAs were extracted using the Apoptotic DNA Ladder Kit (Boehringer Mannheim) according to the manufacturer's instructions, electrophoresed in 2% agarose gel, and stained with 0.5 μg/ml ethidium bromide.

Immunoprecipitation

C2C12 cells were lysed in lysis buffer (1% Brij 96 or Brij 99, 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 1 h at 4°C. Insoluble materials were pelleted at 12,000 rpm for 10 min, and the cell lysates were precleared twice by incubation with protein G (Amersham Pharmacia Biotech) for 30 min at 4°C. Immune complexes were formed by addition of mAbs and collected onto protein G beads, followed by four washes with immunoprecipitation buffer. After elution from beads with Laemmli sample buffer, proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with biotinylated antibody followed by peroxidase-conjugated ExtraAvidin (Sigma Chemical Co.) and visualized with Renaissance Chemiluminescent Reagents (DuPont).

Immunoblotting and Flow Cytometry

Whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and then preblotted with PBS containing 0.05% Tween 20 and 3–5% nonfat dry milk. Proteins were then sequentially immunoblotted with primary antibody followed by peroxidase-conjugated secondary antibody, and visualized with Renaissance Chemiluminescent Reagents.

For flow cytometry, cells were incubated with negative control mAb or specific mAb, washed three times, and then incubated with FITC-conjugated goat anti–mouse Ig. Stained cells were analyzed using a FACScan™ (Becton Dickinson). Fluorescence with negative control mAb was subtracted to give specific mean fluorescence intensity (MFI) units.

Results

Modulation of CD9 Levels and Integrin Association during C2C12 Myogenesis

To evaluate the role of a representative TM4SF protein on myogenic cells, we monitored CD9 expression on mouse C2C12 cells undergoing myogenic cell differentiation. At confluence, C2C12 cells begin fusing into myotubes and express muscle-specific proteins under the control of muscle-specific transcription factors, including MyoD, myogenin, Myf-5, and MRF4 (38). This differentiation is accelerated by medium containing low levels of serum. The level of CD9 protein was upregulated as C2C12 cells approached confluence, and reached a peak at ~1 d after incubation in differentiation medium (Fig. 1, A and B). In contrast, muscle-specific proteins desmin and MHC were upregulated at a later stage. Control antiactin blots showed comparable amounts of protein loaded on each lane (Fig. 1, A and B).

After 1 d of C2C12 myoblast differentiation, TM4SF proteins CD9 and CD81 could be coimmunoprecipitated with several different β1 integrins, including α3β1, α5β1, and α7β1 (not shown). Although there have been many reports of constitutive TM4SF–integrin association (for review see references 24 and 26), there has been little evidence that such complexes can be regulated. Here we show that formation of integrin–CD9 complexes is highly regulated, particularly in the early phase of C2C12 differentiation (Fig. 2 A). During subconfluent growth, relatively little

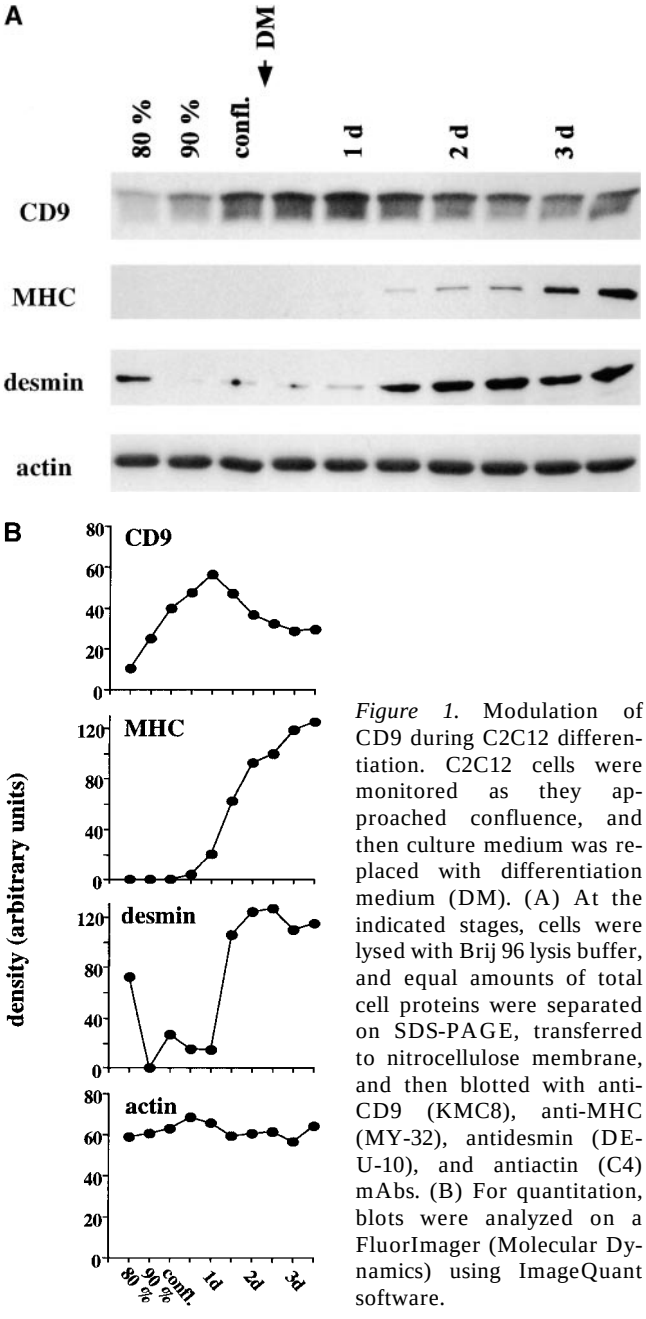

Figure 1. Modulation of CD9 during C2C12 differentiation. C2C12 cells were monitored as they approached confluence, and then culture medium was replaced with differentiation medium (DM). (A) At the indicated stages, cells were lysed with Brij 96 lysis buffer, and equal amounts of total cell proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane, and then blotted with anti-CD9 (KMC8), anti-MHC (MY-32), antidesmin (DE-U-10), and antiactin (C4) mAbs. (B) For quantitation, blots were analyzed on a FluorImager (Molecular Dynamics) using ImageQuant software.

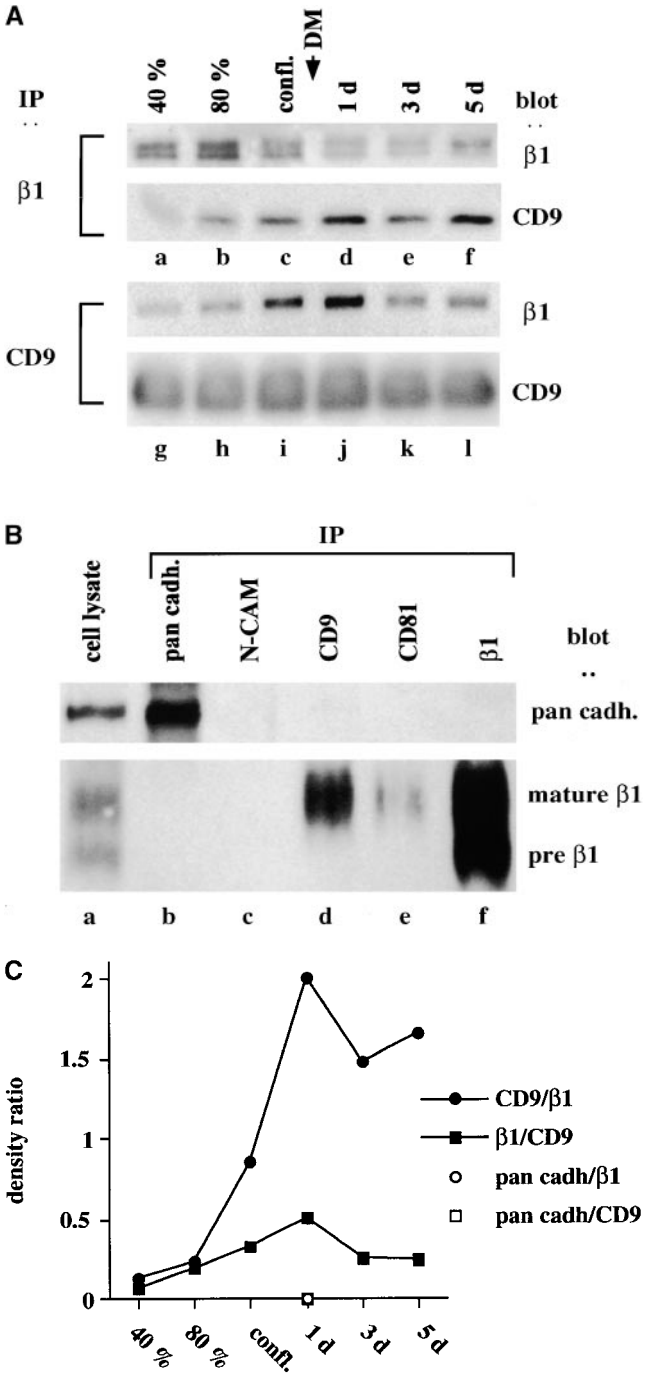

Figure 2. Regulation of integrin–TM4SF complex formation. (A) C2C12 myoblasts were cultured to form myotubes, and cells were lysed in 1% Brij 99 at the indicated stages. Each cell lysate was used for immunoprecipitation (IP) with antiintegrin β1 and anti-CD9 mAbs. After transfer of proteins, membranes were blotted with biotinylated antiintegrin β1 and biotinylated anti-CD9 mAbs in the same experiment. More sample was loaded in lanes g and h to compensate for diminished CD9 expression. (B) Confluent C2C12 myoblasts were lysed and immunoprecipitations were performed using a pan-cadherin mAb (CH-19), and mAbs to N-CAM, CD9, CD81, and integrin β1. Proteins were blotted with biotinylated antiintegrin β1 mAb (KMI6) and pan cadherin mAb (CH-19). (C) For quantitation, blots were analyzed on a FluorImager. Ratios represent coprecipitated CD9 levels/β1 levels (circles, data from A, lanes a–f) and coprecipitated β1 levels/CD9 levels (squares, data from A, lanes g–l).

CD9 was detected in a β1 immunoprecipitation (Fig. 2 A, lane a), and little β1 was present in a CD9 immunoprecipitation (Fig. 2 A, lane g), even though β1 (Fig. 2 A, lane a) and CD9 (Fig. 2 A, lane g) were clearly present. However, as cells reached confluence and began to differentiate, complex formation was clearly upregulated. β1 integrin–CD9 complexes reached a maximum at ~1 d after culture in differentiation medium (Fig. 2 A, lanes a–d and g–j). Complex formation was sustained, or decreased slightly, during the later stages of differentiation (Fig. 2 A, lanes d–f and j–l). Densitometric quantitation of immunoblotted β1 and CD9 levels (Fig. 2 C) confirmed a marked increase in the ratio of β1-associated CD9 relative to total immunoprecipitated β1, with the peak ratio (r = 2.0) occurring after 1 d of differentiation. Likewise, there was an increase

in CD9-associated β1 relative to total immunoprecipitated CD9, again with the peak ratio ($r = 0.5$) occurring after 1 d of differentiation. Notably, these peak ratios were 7–15-fold greater than ratios obtained when cells were 40% confluent. In another experiment, immunoprecipitation of CD81 yielded associated β1 and CD9 proteins, with peak association again occurring at ~1 d after differentiation (data not shown).

In a control experiment, after 1 d of differentiation (Fig. 2 B) mature integrin β1 protein was present in immunoprecipitates of CD9 (Fig. 2 B, lane d) or CD81 (Fig. 2 B, lane e), but not cadherin (Fig. 2 B, lane b) or N-CAM. Both mature and immature precursor forms of β1 were present in C2C12 cell lysate (Fig. 2 B, lane a) and in a β1 immunoprecipitate (Fig. 2 B, lane f).

Effects of Anti-TM4SF and Antiintegrin mAbs on C2C12 Myotube Formation and Maintenance

We used mAbs KMC8 and 2F7 to determine (by flow cytometry) that TM4SF proteins CD9 and CD81 were both present on 100% of C2C12 cells, at levels ~100–1,000-fold above background (data not shown). The addition of mAbs to either CD9 or CD81 caused a marked delay in the formation of myotubes, and both mAbs together showed an additive inhibitory effect, as seen in three different experiments (Fig. 3, A, C, and D). A photo illustrating the delay caused by anti-CD9 plus anti-CD81 mAb at day 3 is shown in Fig. 4 A. In contrast to the anti-TM4SF antibodies, antiintegrin anti-α4, anti-α5, and anti-β1 antibodies did not delay myotube formation. Likewise, no de-

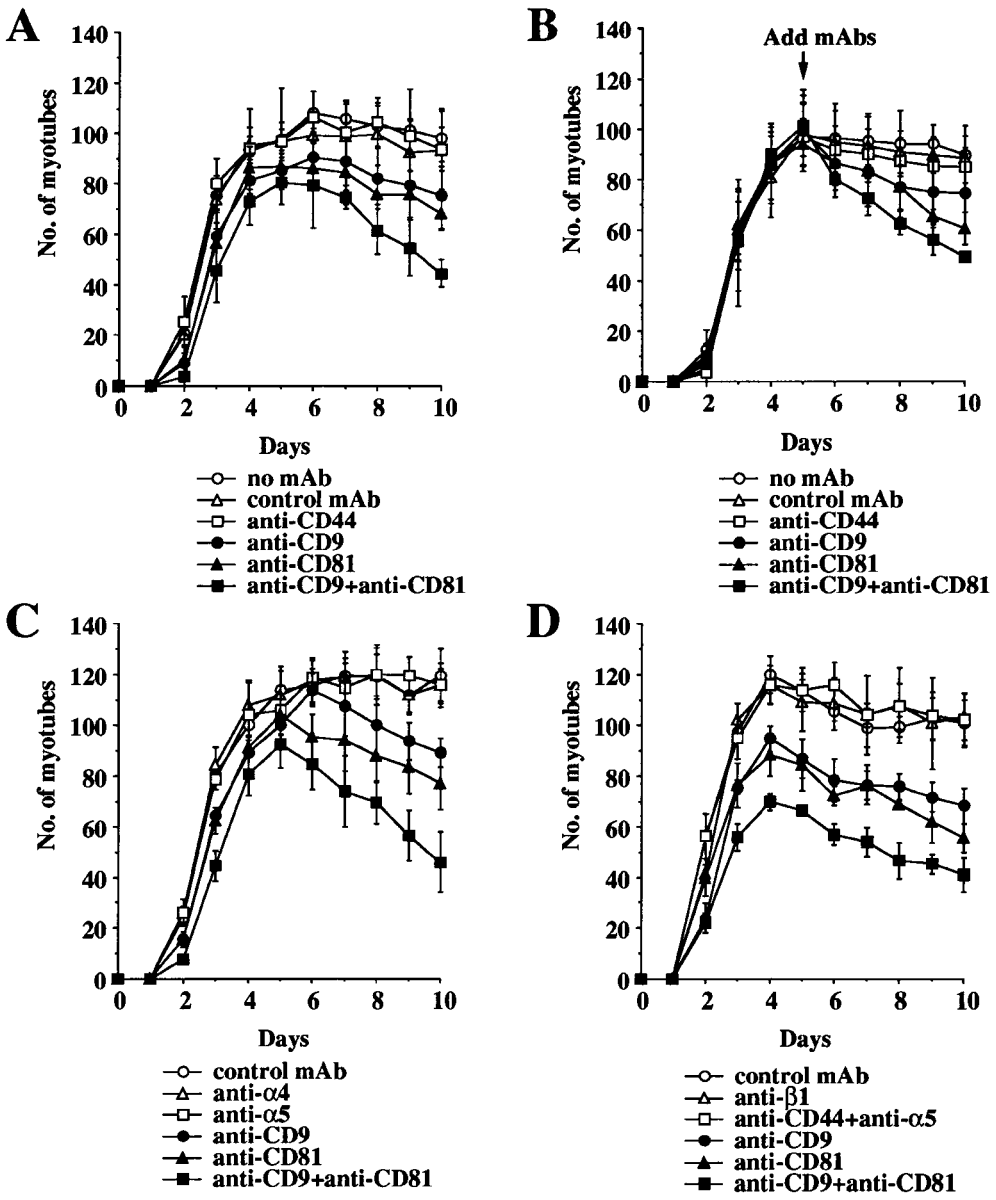

Figure 3. Anti-TM4SF mAbs affect C2C12 myotube formation. C2C12 myoblasts were cultured in a 96-well tissue culture plate. (A, C, and D) When cells were confluent (day 0), medium was replaced with differentiation medium containing 10 μg/ml of various mAbs. In B, mAbs were not added until 5 d after medium replacement. mAbs used were KM114, anti-CD44; KMC8, anti-CD9; 2F7, anti-CD81; R1-2, antiintegrin α4; MFR5, antiintegrin α5; Ha2/5, antiintegrin β1; and R35-95, negative control. In several wells, two mAbs (10 μg/ml for each) were combined. Each data point represents the mean number of myotubes from four determinations (from independent 2.6-mm^2 fields) ±SD. Flow cytometry and immunoprecipitation showed that CD9, CD81, CD44, and integrin α5 were all expressed comparably in confluent C2C12 myoblasts, whereas integrin α4 was expressed only in C2C12 myotubes (data not shown). Also, all antibodies (except R35-95) used here and in Fig. 4 were shown previously to inhibit cell functions in vitro. For example, KM114 (reference 48), R1-2 (reference 19), MFR5 (reference 23), and Ha2/5 (reference 46) inhibited cell adhesion, whereas KMC8 and 2F7 inhibited myeloid and T cell development, respectively (references 10, 53).

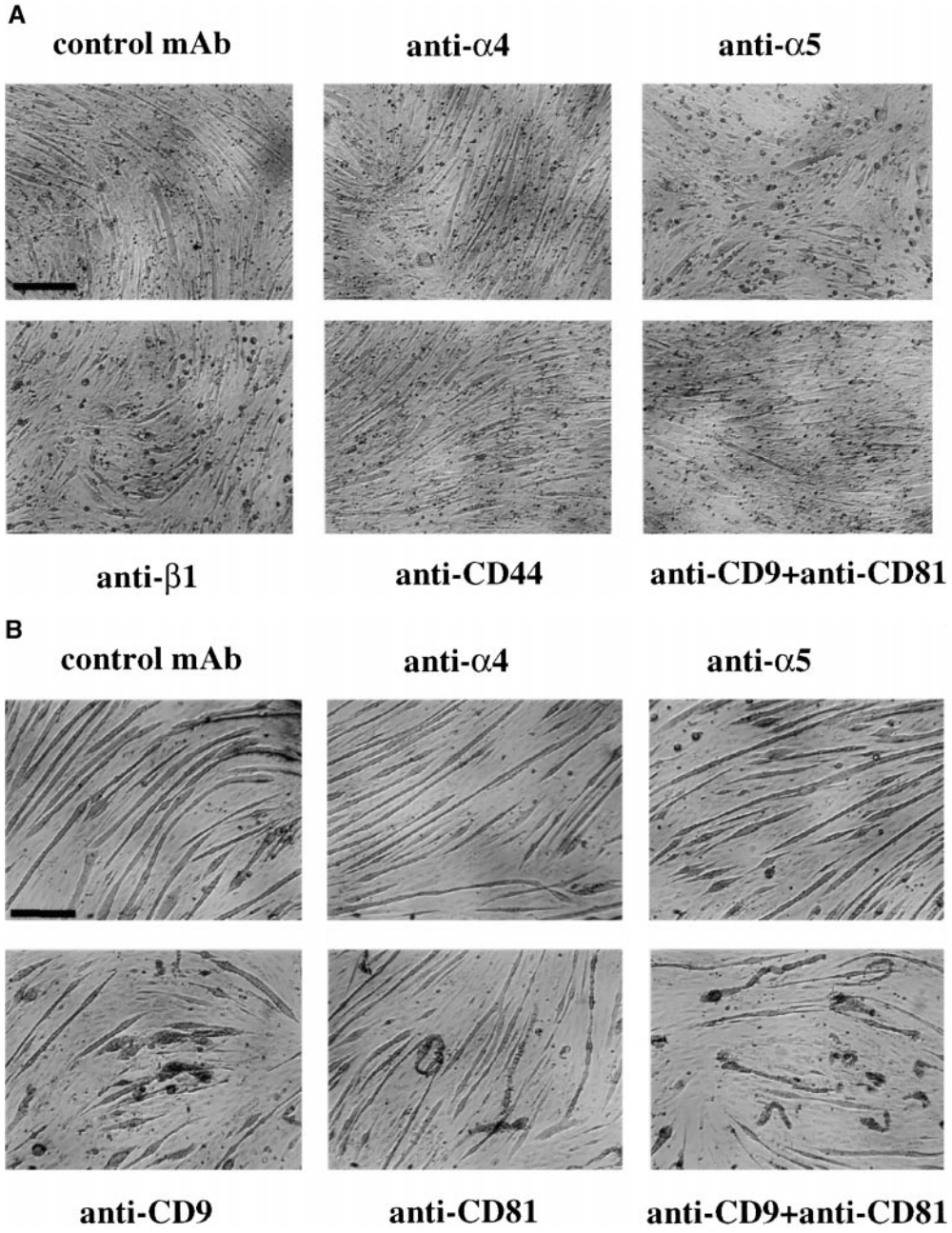

Figure 4. Delay of myotube formation (A) and early degeneration of myotubes (B) in the presence of anti-TM4 mAbs. C2C12 cells were differentiated in the presence of 10 μg/ml of various mAbs as in the legend to Fig. 3. Cell images at day 3 (A) and day 10 (B) were saved using a light microscope and the computer program, Scion Image 1.60. Bars, 250 μm.

lay was caused by anti-CD44 or anti-CD44 plus anti-α5 together (Fig. 4 A and Fig. 3, C and D). However, anti-α5 and anti-β1 mAbs did cause myotubes at day 3 to be significantly shorter than myotubes treated with control mAb, anti-CD44 mAb, or anti-α4 mAb (Fig. 4 A). mAbs to other integrin subunits were not tested for the following reasons: an anti-murine α3 mAb is not yet available, the anti-α7 mAb was not available in sufficient quantity, and the α6 subunit is only weakly expressed on C2C12 cells. Anti-CD9 and anti-CD81 antibodies, either alone or in combination, had no effect on myoblast proliferation as C2C12 cells grew to confluence in growth medium over a 5-d period (data not shown). Thus, anti-TM4SF antibody effects on myotube formation are not an indirect consequence of inhibition of myoblast proliferation.

Anti-CD9 and anti-CD81 mAbs not only caused a delay in myotube formation, but also accelerated myotube degeneration (Fig. 3). Specific examples of myotube degradation at day 10 are shown in Fig. 4 B. Again, the effects of anti-CD9 and anti-CD81 mAb were additive (Fig. 3 and Fig. 4 B). Again, myotube maintenance was not affected by control mAb, anti-α4, anti-α5, anti-CD44, or anti-β1 mAbs (Fig. 3 and Fig. 4 B). In Fig. 3 B, mAbs were not added to cultures until day 5, when peak numbers of myotubes were already formed. Nonetheless, in subsequent days the numbers of myotubes again were diminished upon addition of anti-TM4SF mAb, with the effects of anti-CD9 and anti-CD81 mAbs being additive. This result excludes the possibility that delayed myotube formation is responsible for the subsequent degeneration of myotubes.

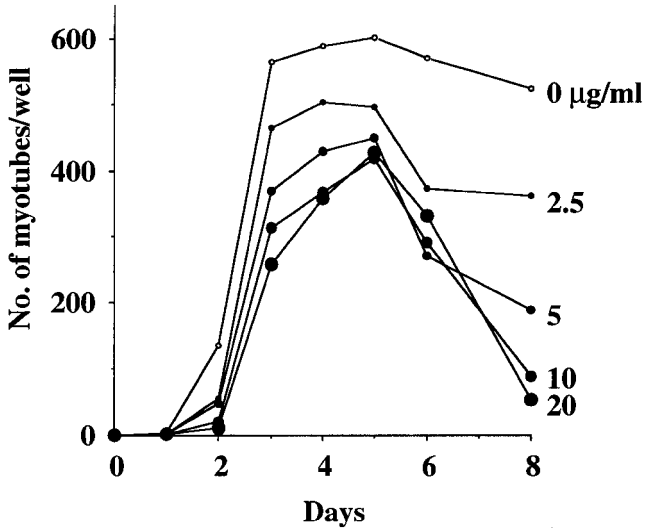

Figure 5. Dose-dependent inhibition of elongated myotube formation by anti-CD9 mAb. Confluent C2C12 cells were incubated with differentiation media (day 0) in the presence of various concentrations of anti-CD9 mAb (KMC8). Numbers of myotubes longer than 250 μm were determined by counting entire wells (~38 mm^2) of a 96-well plate. Thus, even though only long myotubes were counted, larger numbers are obtained than in Fig. 3, in which fields of 2.6 mm^2 were analyzed. Myotube length was determined using the program Scion Image 1.60. For each point, $n = 1$.

Effects of an anti-CD9 mAb (KMC8) were even more dramatically obvious when elongated myotubes (>250 μm) were analyzed rather than total myotubes. As shown in Fig. 5, ~50% fewer elongated myotubes were formed in the presence of 20 μg/ml KMC8 at day 3. By day 5, the inhibitory effect was less pronounced, with ~25% fewer elongated myotubes being formed. The most dramatic effect of mAb KMC8 was seen after maximal elongated myotube formation had already occurred (days 6–8). For example, at day 8, ~90% fewer myotubes were maintained when 10–20 μg/ml KMC8 was present (Fig. 5). These anti-CD9 mAb effects were clearly dose dependent.

To assess antibody effects on myotube attachment, C2C12 myoblasts were allowed to differentiate into myotubes in the presence of antibodies for 5 d, and then detached myotubes were removed by washing. Counting of myotubes present before and after washing (in quadruplicate) revealed that myotube detachment was relatively unaffected by antibody incubation (no antibody, 29% detachment; control IgG, 27% detachment; anti-α4, 32% detachment; anti-α5, 34% detachment; anti-CD44, 33% detachment; anti-CD9, 30% detachment; anti-CD81, 31% detachment; anti-CD9 plus anti-CD81, 18% detachment). Thus, failure to maintain myotubes did not appear to result from direct promotion of detachment by anti-CD9 or anti-CD81 mAbs. In another experiment, neither anti-CD9 nor anti-CD81 mAb had any effect on confluent C2C12 myoblast cell adhesion to tissue culture plastic, or to surfaces coated with fibronectin or mouse laminin 1. Likewise, these antibodies had no consistent effect on either random C2C12 cell motility or transwell haptotaxis towards fibronectin or laminin (data not shown). In a separate experiment, a culture of confluent myoblasts was disrupted by scratching with a plastic tip. Again, anti-CD9 and anti-CD81 antibodies had no effect on the rate of myoblast migration into the vacated area, or on the alignment of myotubes in that area.

Anti-TM4SF mAbs Do Not Delay MHC or Desmin Expression

A previous report demonstrated that antiintegrin β1 mAb, CSAT, inhibited both morphological differentiation (myotube formation), and biochemical differentiation (meromyosin expression) in chicken embryo myoblasts (47). Here we demonstrate that a combination of anti-TM4SF CD9 and CD81 mAb did not alter biochemical differentiation of C2C12 cells (Fig. 6), marked by the appearance of either MHC or desmin. In contrast, an anti-β1 mAb did substantially lower the appearance of MHC, consistent with previous anti-β1 mAb effects on biochemical differentiation (47). Control antiactin immunoblots showed that equal amounts of protein were loaded in each lane.

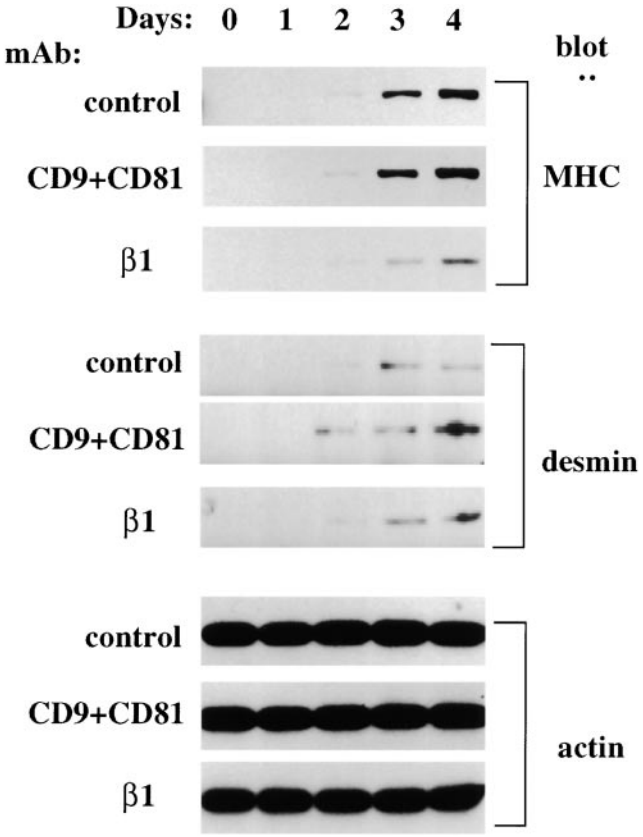

Figure 6. C2C12 cell expression of MHC and desmin is not affected by anti-TM4SF mAbs. C2C12 cells were differentiated in the presence of 10 μg/ml of control, antiintegrin β1, or anti-CD9 + anti-CD81 mAbs. Cells were lysed with Brij 96 lysis buffer 0–4 d after culture in differentiation medium (DM), and whole cell lysates were fractionated on SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and then blotted with anti-MHC, antidesmin, and antiactin mAbs.

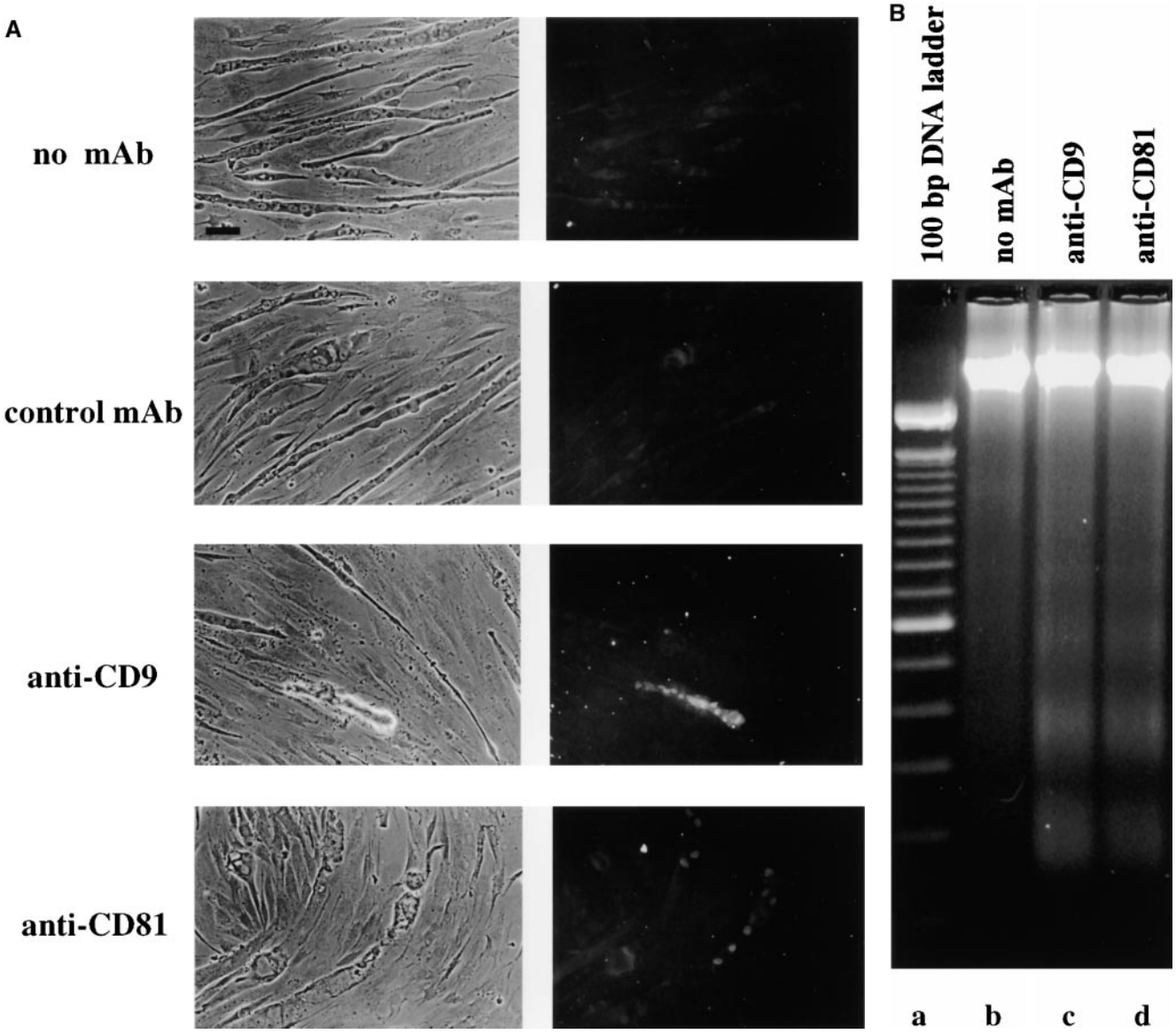

Figure 7. Induction of C2C12 myotube apoptosis by anti-TM4SF mAbs. (A) C2C12 cells were cultured in differentiation medium for 6 d on a slide precoated with mouse laminin in the absence or presence of control, anti-CD9, and anti-CD81 mAbs. Cells were then fixed (2% paraformaldehyde) and permeabilized (0.1% Triton X-100), and nuclei of apoptotic cells were stained by TUNEL. Cells were visualized using a Zeiss Axioscop microscope, and phase–contrast (left panels) and epifluorescent (right panels) images were obtained. Bar, 50 μm. (B) C2C12 cells were again differentiated for 6 d in the absence or presence of anti-TM4SF mAbs. Then, myotubes were enzymatically separated from unfused myoblasts, and DNAs were extracted, electrophoresed in 2% agarose gel, and stained with ethidium bromide. Lane a shows a control 100-bp DNA ladder.

Anti-TM4SF mAbs Cause Early Apoptosis of C2C12 Myotubes

Anti-CD9 and anti-CD81 mAbs induced early degeneration of C2C12 myotubes (Figs. 3–5). Some of these myotubes showed cellular blebs (data not shown), which is suggestive of apoptosis. Thus, we used the TUNEL method to confirm whether degenerating myotubes were apoptotic. Fluorescein-labeled UTP was incorporated into nuclei of degenerating myotubes after 6 d in the presence of anti-CD9 or anti-CD81 mAb (Fig. 7 A, right panels). In contrast, myotube nuclei were not stained when either control mAb or no mAb was present. Also, no unfused myoblasts were stained in any of the cultures. Because only a fraction of the total myotubes appeared to undergo apoptosis, we did not expect to see a strong DNA laddering effect. Nonetheless, we separated myotubes from unfused myoblasts, extracted DNA, and studied apoptosis-associated internucleosomal fragmentation by electrophoresis (Fig. 7 B). Albeit at a low level, DNA laddering was evident in myotubes cultured in the presence of anti-CD9 or anti-CD81 mAb (Fig. 7 B, lanes c and d). In contrast, the control lane did not show DNA laddering although the same amount of DNA was loaded (Fig. 7 B, lane b).

In additional studies, apoptosis in a mixed myotube/

myoblast preparation was determined by measuring histone-associated DNA fragments (Cell Death Detection ELISA^{PLUS} Kit; Boehringer Mannheim). After only 4 d of differentiation, anti-CD9 and anti-CD81 mAb each yielded ~1.1–1.2-fold enhancement of apoptosis, and both antibodies together yielded only ~1.5-fold enhancement of apoptosis. However, after 6 d of differentiation, anti-CD9 and anti-CD81 mAb each yielded ~1.5-fold enhancement of apoptosis (compared with control mAb), and both antibodies together yielded ~3-fold enhancement of apoptosis. Together these results suggest that anti-TM4SF mAbs induce early apoptosis of C2C12 myotubes, but not unfused myoblasts.

CD9 Promotes RD Cell Syncytia Formation

For further evaluation of TM4SF protein function in muscle-derived cells, we transfected CD9 into the human myoblast-derived RD sarcoma cell line. Although confluent RD cells continue growing, they constitutively express myogenic transcription factors, and undergo a limited and abortive myogenic differentiation (11, 28, 65). Here we analyzed RD cell transfectants as they became confluent after ~6 d in 2% FCS. An RD cell line overexpressing CD9 (RD-C9-2) became substantially more multinucleate than untransfected RD (Fig. 8, left panels). Likewise, RD cells transfected with CD9 plus integrin α3 subunit (RD-A3C9) became substantially more multinucleate than RD cells transfected with α3 alone (RD-A3; Fig. 8, right panels). Some of these multinucleate cells were myotubes, but most of them were apolar, giant cells that resemble virus-induced syncytia (Fig. 8, bottom panels). Using time-lapse video microscopy, we confirmed fusion between a multinucleate giant cell and a mononucleate cell (data not shown). Despite differences in syncytia formation, all of the RD transfectants proliferated at essentially the same rate as RD cells.

Quantitation revealed that two distinct RD lines overexpressing CD9 (RD-C9-1, RD-C9-2) formed approximately fourfold more syncytia than untransfected, mock-transfected (RD-Z), or control transfected (RD-A3) cells (Fig. 9 A). Also, two distinct RD lines expressing both α3 integrin and CD9 (RD-A3C9-1, RD-A3C9-2) showed approximately eightfold more syncytia than control RD cells, including RD-A3 cells expressing α3 alone (Fig. 9 A). CD81 overexpression studies were not carried out because CD81 is already highly expressed on RD cells. Effects of TM4SF overexpression on longer-term syncytia maintenance could not be determined, as syncytia became obscured and displaced by unfused tumor cells that were continually growing and preferentially adherent. In a separate experiment, CD9-transfected fibrosarcoma cells (HT1080-CD9) (6) were cultured for 5–8 d as they became confluent in 2% FCS. No syncytia were observed for this cell line during this time.

Anti-CD9 mAbs, BU16 and DU-ALL-1 (Fig. 9 B), substantially delayed the appearance of RD-C9-1 cell syncytia. However, no delaying effect was seen with another anti-CD9 mAb, C9/BB, which recognizes a different CD9 epitope. An anti-CD81 mAb, M38, also delayed syncytia formation, whereas a control antiintegrin α5 mAb, A5-PUJ2, did not affect syncytia formation.

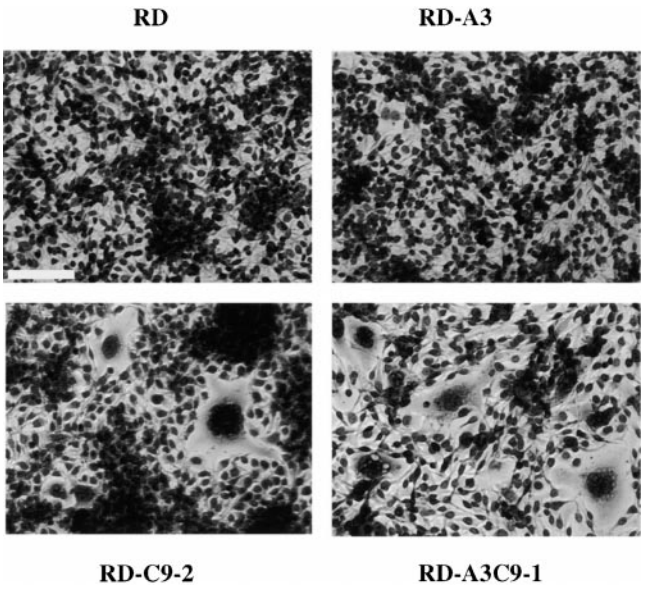

Figure 8. Syncytia formation by RD transfectants. RD transfectants (2×10^4) were plated into wells of a 96-well plate and cultured in DME containing 2% FCS for 6 d until confluence. Cells were visualized using Wright stain, and the program Scion Image 1.60 was used to acquire and analyze computer images from a Zeiss Axiovert 135 microscope. Levels of CD9, in MFI units, were 31 (RD), 10 (RD-A3), 1122 (RD-C9-2), and 910 (RD-A3C9-1). Levels of α3 were 10 (RD), 67 (RD-A3), 11 (RD-C9-2), and 66 (RD-A3C9-1). Bar, 250 μm.

Discussion

Despite the strong expression of CD9, CD81, and other TM4SF proteins on skeletal muscle (50, 58), the role of TM4SF proteins during myogenesis had not been studied previously. Here we found that CD9 and CD81 are also abundant on murine myoblast C2C12 cells, and we provide strong evidence for the involvement of at least two TM4SF proteins (CD9 and CD81) during myoblast differentiation. First, CD9 expression was upregulated during the early phase of myogenic differentiation of murine C2C12 cells. Second, anti-CD9 and anti-CD81 antibodies substantially delayed fusion of C2C12 myoblast cells and RD rhabdomyosarcoma cells. Third, CD9 overexpression promoted cell fusion in four independently transfected myoblast-derived RD cell lines.

Possible Mechanisms for TM4SF Protein Contributions to Myoblast Fusion

In contrast to muscle-specific proteins MHC and desmin, CD9 expression was upregulated at a much earlier stage of myoblast differentiation. In addition, anti-TM4SF antibodies caused a delay in cell fusion without altering biochemical differentiation of C2C12 cells (as evidenced by a lack of effect on MHC or desmin expression). Thus, CD9 expression appears neither to participate in myogenic transcription factor regulation of other proteins, nor to be regulated by myogenic transcription factors. Also, the delay in fusion seen here was not due to altered myoblast proliferation, although antibodies to CD9 and CD81 have

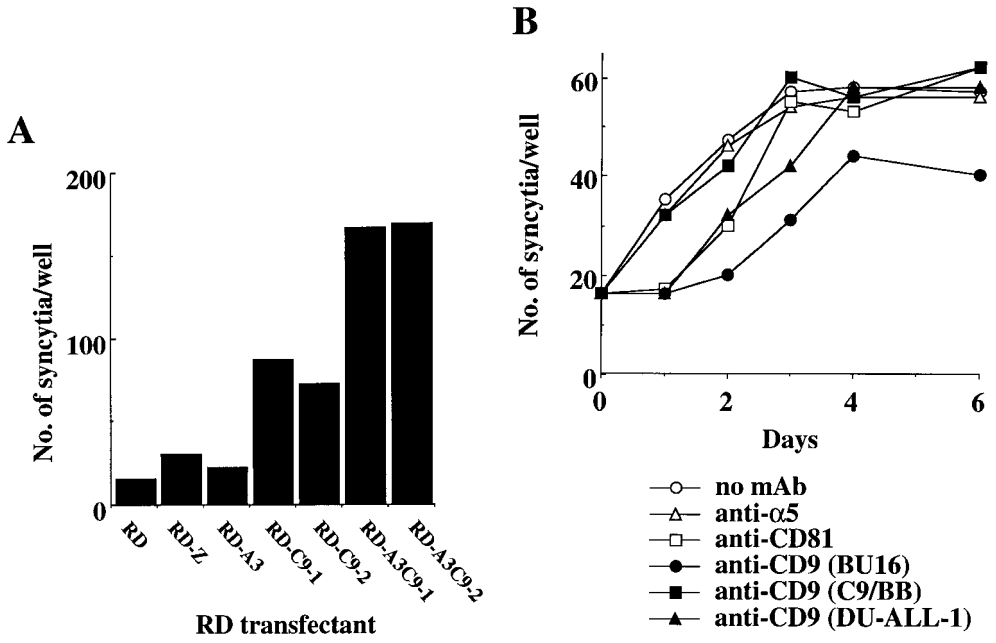

Figure 9. Quantitation and inhibition of RD cell syncytia formation. (A) RD transfectants were cultured, and then syncytia (with more than three nuclei) were counted daily, until confluence. Maximal numbers of syncytia for each transfectant are shown. RD-Z corresponds to mock (pZeoSV)-transfected RD. Coimmunoprecipitation experiments confirmed that integrin α3β1 associated with CD9 in the RD-A3C9 transfectants (data not shown). RD-C9-1 and RD-C9-2 were independently derived from RD cells; RD-A3C9-1 and RD-A3C9-2 were independently derived from RD-A3 cells. (B) RD-C9-1 cells were cultured for 6 d in the absence or presence of mAb to integrin α5 (A5-PUJ2), CD9 (BU16, DU-ALL-1, and C9-BB), or CD81 (M38). Similar results were obtained in multiple additional experiments using both RD-C9-1 and RD-C9-2 cells (data not shown). The BU16 (39) and M38 (20) antibodies were shown previously to inhibit virus-induced syncytia. Notably, the levels of α5 (∼60 MFI units) and CD81 (∼300 MFI units) were essentially unchanged in the various RD cell transfectants. For each reported data point, $n = 1$.

been reported to affect lymphocyte proliferation (62, 67). Likewise, delayed fusion did not appear to arise from promotion of cell detachment, inhibition of attachment, or altered C2C12 cell migration or alignment. Instead, we hypothesize that TM4SF proteins may delay or inhibit myogenesis at an early stage, by a mechanism that may directly influence cell fusion.

Fusion was delayed in two different cell types, upon treatment with three different anti-CD9 mAbs and two different anti-CD81 mAbs. In addition, RD cell fusion was promoted upon transfection of CD9. These results are consistent with TM4SF proteins contributing to fusion, perhaps by engaging in protein–protein interactions with each other, or with other proteins. In this regard, TM4SF proteins are reported to associate laterally with many other cell surface proteins (24, 40, 61, 73). At present, there is no evidence for TM4SF proteins having counter-receptors that would participate in cell–cell interactions. Although CD9 (and α3 integrin) promoted fusion in RD cells, no syncytia formation was seen in CD9-transfected HT1080 fibrosarcoma cells, which are known to contain CD9–α3β1 integrin complexes (6). Thus, CD9 and α3 integrin by themselves are not sufficient to promote fusion, as the rhabdomyosarcoma RD cells must contain specific proteins or other factors required for cell fusion.

The TM4SF effects on myoblast fusion shown here are reminiscent of anti-TM4SF antibody effects and CD9 overexpression effects on virus-induced syncytia formation (17, 20, 39, 72). For example, the delay in myoblast fusion seen here with anti-CD81 and anti-CD9 antibodies could be mechanistically related to the delay in feline immunodeficiency virus (FIV) production caused by anti-CD9 mAb (17). Also, a more distantly related TM4SF protein called peripherin/rds was reported to promote fusion of lipid vesicles in vitro (9). Besides directly contributing to fusion through potential protein–protein interactions, TM4SF proteins might also contribute to myoblast fusion by regulating cellular signaling. In this regard, CD9 and CD81 associate with phosphatidylinositol 4-kinase (7) and with PKCα and PKCβII (Zhang, X., and M. Hemler, manuscript in preparation). Consistent with this, PKC plays a key role in myoblast fusion (16), and PKCα is present in both myoblasts and myotubes (29).

TM4SF Proteins and Integrins

We found that CD9 on C2C12 cells associates with various β1 integrins, including α3β1, α5β1, and α7β1 and that CD9 associated with α3β1 on α3-transfected RD cells. These results are consistent with integrin–TM4SF protein associations seen on many other cell lines (6, 22, 24, 26, 33, 40, 51, 52). Importantly, we have provided firm evidence for the upregulation of CD9–β1 integrin complexes in coordination with the onset of myoblast differentiation. As far as we know, there have been few if any prior demonstrations of physiologically relevant integrin–TM4SF complex regulation. The regulated formation of TM4SF–integrin complexes suggests that they could play a role during myogenesis. Indeed, even though α3 integrin alone did not effect RD cell fusion, it potentiated the effects of CD9, such that twice as many syncytia were formed. Possibly, integrin expression could contribute to myoblast fusion by altering the distribution and/or signaling functions of CD9 in a way that does not affect integrin-dependent cell adhe-

sion or motility. The α3β1 integrin is absent from adult striated muscle, but is variably present in fetal skeletal muscle (4, 43, 44), whereas the α7β1 and α5β1 integrins are prominent on myoblasts and developed skeletal muscle (14, 37, 59, 60).

Although integrins may modulate TM4SF protein contributions during myogenesis, there are several critical differences between the roles of TM4SF proteins and integrins. First, anti-CD9 and anti-CD81 antibodies caused a delay in myoblast fusion, whereas antiintegrin α4, α5, and β1 antibodies did not cause a delay in the appearance of the peak number of myotubes. Second, anti-α5 and anti-β1 antibodies caused myotubes at day 3 to be significantly shortened, but anti-CD9 and anti-CD81 did not have this effect. Third, anti-CD9 and anti-CD81 mAbs had no effect on biochemical differentiation of C2C12 cells (defined by MHC or desmin appearance), whereas an anti-β1 mAb did markedly inhibit MHC appearance, consistent with previous anti-β1 mAb effects on biochemical differentiation in chicken embryo myoblasts (47). Fourth, antiintegrin antibodies may affect myogenesis largely by blocking myoblast cell adhesion and/or motility (15, 32, 47, 77). In contrast, we have not found anti-TM4SF antibodies, or CD9 overexpression to have an effect on cell adhesion here or elsewhere (e.g., see references 6, 41, 78). Likewise, we saw no effect of anti-CD9 or anti-CD81 mAbs on C2C12 cell motility, despite reports showing TM4SF proteins contributing to the motility of other cell types (2, 31, 49, 75, 78). In conclusion, integrins may associate with TM4SF proteins and modulate their functions during myogenesis, but for the most part their specific functional contributions appear to be very distinct.

Apoptotic Degradation of Myotubes

Aside from CD9 costimulation of T cells leading to apoptosis (63), there have been few reports linking TM4SF proteins to apoptosis. Here we used three different methods to demonstrate that anti-CD9 and anti-CD81 antibodies promoted apoptotic degradation of C2C12 myotubes after they were formed. The antibodies did not trigger myoblast apoptosis. Also, if anti-CD9 and CD81 mAbs were not added until myoblast fusion had already occurred, they still resulted in apoptotic degradation. Thus, delayed cell fusion was not a prerequisite for apoptotic degradation. Conversely, apoptotic degradation did not contribute to a delay in cell fusion. The delay in fusion was most obvious during days 1–3 of differentiation, whereas apoptotic degradation was not obvious at day 3 (see Fig. 3 and Fig. 4 A) and was not readily detectable until at least day 5.

In previous reports, myotube degradation and apoptosis in vitro and in vivo were associated with disrupted expression and localization of integrin α7β1, and loss of adhesion to laminin-2/4 (42, 68, 69). In vivo muscle cell degradation, possibly due to diminished cell adhesion, also occurred in skeletal muscle containing high numbers of cells lacking α5 (66). However, because neither CD9 nor CD81 appears to regulate myotube adhesion, or to promote myotube detachment, they must regulate apoptosis by a different mechanism, perhaps by modulating cell signaling (as discussed above). Although myoblast precursors may be more generally susceptible to apoptosis than terminally differentiated myotubes (70), we did not observe unfused myoblast apoptosis resulting from anti-CD9 and anti-CD81 mAb treatment. Thus, TM4SF protein modulation of apoptosis appears to apply selectively to myotubes, and not myoblasts.

Other Cell Surface Proteins Involved in Myogenesis

Other cell surface proteins such as N-cadherin, M-cadherin, N-CAM, meltrin, VCAM, and CDO have also been implicated in the process of myoblast fusion (see Introduction). However, in comparison to CD9 and CD81, perturbation of these other proteins causes not just a delay, but a more substantial inhibition of myoblast fusion. In addition, these other proteins have not been linked to apoptotic degradation of myotubes. Although antibodies to N-cadherin, α4 integrin, and β1 integrin inhibited myoblast fusion, deletion of these genes did not adversely affect muscle formation (13, 27, 76). Thus, contributions of each of these proteins could be compensated by the functions of other related proteins.

It is well established that TM4SF proteins including CD9, CD63, CD81, CD82, and CD151 can form complexes with each other within cellular membranes (1, 6, 31a). Thus, besides CD9 and CD81, some of these other TM4SF proteins might possibly also be involved in myoblast fusion. However, definitive analysis of the role of other TM4SF proteins on C2C12 cells will not be readily achieved until the appropriate antimurine mAbs become available. It remains to be determined whether deletion of genes for any TM4SF proteins will have an adverse effect on myoblast fusion in vivo. In this regard, there were no abnormal phenotypes at birth in muscular systems of CD81-deficient mice (Tsitsikov, E.N., J.C. Gutierrez-Ramos, and R.S. Geha, unpublished observations). Given that several different TM4SF proteins may form complexes with each other, it seems highly possible that loss of one particular TM4SF protein may be compensated by others.

In conclusion, we have shown that TM4SF proteins CD9 and CD81 may play key roles during myoblast fusion, as seen in both murine C2C12 cells and human RD cells. Furthermore, CD9 and CD81 may also contribute to the protection of myotubes from apoptosis.

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