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NCAM(CD56) and RUNX1(AML1) Are Up-Regulated in Human Ischemic Cardiomyopathy and a Rat Model of Chronic Cardiac Ischemia

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Chronic myocardial ischemia is the leading cause of impaired myocardial contractility and heart failure. To identify differentially expressed genes in human ischemic cardiomyopathy (ICM), we constructed a subtracted cDNA library using specimens of ICM compared to normal human heart. Among 100 randomly sequenced clones, seven sequences represented recently identified candidate genes for differential expression in cardiac hypertrophy. A further clone without a known hypertrophy-association coded for the adhesion molecule NCAM(CD56). RNase protection assay, immunohistochemistry, and Western blotting revealed strong overexpression of NCAM(CD56) in all hearts with ICM (n = 14) compared to normal hearts (n = 8), whereas in congestive cardiomyopathy (CCM) (n = 8), hypertrophic obstructive cardiomyopathy (n = 2), myocarditis (n = 4), and sarcoidosis (n = 2), at most slight overexpression of NCAM(CD56) was observed. NCAM(CD56) overexpression abnormally involved the whole cell membrane and the cytoplasma of cardiomyocytes only inside and adjacent to ischemia-induced cardiac scars. Normal or hypertrophic fibers at a distance from ischemic scars were devoid of NCAM overexpression. Identical alterations were observed in an experimental rat ICM model, but not in normal nor in spontaneously hypertensive rat hearts. In search of NCAM(CD56)-related transcription factors we found RUNX1(AML1) up-regulation in ICM and detected RUNX1(AML1) binding within the NCAM(CD56) promoter by electromobility shift assay. We concluded that strong overexpression of NCAM(CD56) and RUNX1(AML1) is a constant and characteristic feature of cardiomyocytes within or adjacent to scars in ICM. *(Am J Pathol 2003, 163:1081–1090)*

The most common cause of chronic heart failure is coronary artery disease (CAD), which results in left ventricular dysfunction.[1,2] The morphological changes of the heart in chronic heart failure due to CAD have been termed ischemic cardiomyopathy (ICM).[1,3–6]

Among the earliest events during ischemia-induced ventricular dysfunction, the renin-angiotensin system and secretion of atrial natriuretic peptide (ANP) are activated.[7,8] In addition, in the endothelin system,[9,10] cytokines such as IL-1, IL-6, and tumor necrosis factor-α,[11–14] stress-proteins,[15] and anti-oxidants[16] change their expression pattern. However, these changes generally are not characteristic for ICM.

To identify differentially overexpressed genes in ICM compared to normal hearts we used a PCR-based technique to construct a subtracted cDNA library. We find that strong overexpression of NCAM (CD56) and the transcription factor RUNX1(AML1) is a highly sensitive and characteristic marker of cardiomyocytes within or adjacent to scars in ICM compared to normal hearts, while at most slight overexpression is observed in CCM, hypertrophic obstructive cardiomyopathy (HOCM), and myocarditis, including sarcoidosis. This molecular response to ischemic heart damage appears to be phylogenetically conserved, because analogous alterations occurred in an experimental rat model of ischemic heart disease compared to normal and spontaneously hypertensive rats.

Materials and Methods

Human Tissue

Heart tissue obtained from autopsies within 6 hours after death was shock-frozen in liquid nitrogen and stored at −80°C. Ischemic heart disease of the 14 patients in this study had been diagnosed either by coronary angiography (n = 8), medical history of myocardial infarction with typical electrocardiogram signs (n = 3), or by clinical features of acute myocardial infarction (n = 3). In the eight patients with congestive cardiomyopathy (CCM)

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Table 1. Clinical and Pathological Data of Patients Investigated (n = 28)

Case no.	Sex/ age	Clinical and pathological diagnoses	Heart weight
188/95	M/63	AH, severe CA with two VD, recurrent MI	620 g
313/95	F/62	AH, recurrent MI, severe CA with three VD	580 g
S233/96	M/68	DM, AH, severe CA with three VD, liver cirrhosis, septicopyemia	520 g
S234/96	M/88	AH, severe CA with three VD, bronchopneumonia	550 g
S280/96	M/68	AH, recurrent MI, severe CA with three VD	720 g
S130/97	M/78	AH, recurrent MI, severe CA with two VD (RCX, RCA)	680 g
S190/98	M/45	AH, recurrent MI, severe CA with three VD	510 g
S196/98	M/70	AH, severe CA with two VD, recurrent MI	440 g
S236/98	M/63	AH, DM, recurrent MI, severe CA	700 g
S252/98	M/69	DM, recurrent MI, severe CA with two VD (LCA, RCA)	450 g
S258/99	M/63	AH, DM, acute MI, severe CA, recurrent MI	530 g
S7/99	M/74	Recurrent MI, severe CA with three VD	310 g
S52/99	M/51	AH, recurrent MI, moderate CA	440 g
S04/00	F/60	AH, recurrent MI, moderate CA	680 g
S111/97	M/62	CCM, no CA	710 g
E22500/90	M/54	CCM, no CA	630 g
S141/97	M/32	CCM, no CA	570 g
S100/99	F/59	CCM, no CA	680 g
S18/00	F/57	CCM, no CA	570 g
S254/99	M/71	CCM, no CA	720 g
S132/00	M/44	CCM, no CA	650 g
S208/01	M/67	CCM, no CA	860 g
S183/99	M/33	HOCM, no CA	720 g
S196/93	M/60	HOCM, no CA	490 g
A15076/01	F/36	Sarcoidosis, no CA	440 g
A15051/01	M/51	Sarcoidosis, no CA	360 g
A17995/02	M/7	Parvovirus B19 myocarditis, no CA	NA
A12978/01	M/71	Eosinophilic myocarditis, no CA	680 g
A12021/01	F/39	Giant cell myocarditis, no CA	450 g
S 256/99	M/31	Giant cell myocarditis, no CA	320 g
S244/96	F/24	Moschcowitz syndrome, no CA	250 g
S55/97	F/61	Rupture of arteriovenous angioma, AH, no CA	360 g
S31/98	F/52	Craniocerebral trauma, no CA	280 g
S62/98	M/43	Anaphylactic shock, no CA	300 g
S200/98	M/61	Malignant melanoma, no AH, no CA	290 g
S114/99	M/26	Anaphylactic shock, no CA	270 g
S160/99	M/29	Aplastic anemia, liver dystrophy, no CA	280 g
S133/00	F/63	Adenocarcinoma of the ovary, no CA	270 g

AH, Arterial hypertension; CA, Coronary arteriosclerosis DM, Diabetes mellitus; MI, Myocardial infarction; VD, Vessel disease;

and the two patients with HOCM, absence of coronary artery disease was angiographically confirmed. Normal human hearts (n = 8) as well as heart specimens from patients with myocarditis (n = 4) and sarcoidosis (n = 2) were without a history or autopsy findings suggestive of cardiovascular disease and hypertension. The study was approved by an institutional review committee, with all procedures following institutional guidelines.

Animal Tissue

Myocardial infarction was induced by ligation of the left coronary artery in adult (250 to 290 g) female Wistar rats (Charles River, Sulzfeld, FRG) as described.[17] Sham-operated rats served as controls. Eight weeks after surgery, infarcted (n = 6) and sham-operated hearts (n = 6) were removed. Spontaneously hypertensive rats (SHR; Charles River) (310 to 330 g) were used as non-ischemic cardiomegaly controls. Samples were fixed in 4% paraformaldehyde, sliced perpendicular to the long axis of the heart, and embedded in paraffin.

Subtracted cDNA Library

2 μg of mRNA from an ICM (case number S280/96) and a normal heart (case S62/98) (Table 1) were used for polymerase chain reaction (PCR)-based cDNA subtraction (PCR-Select Subtraction kit; Clontech, Heidelberg, Germany) following the manufacturer's instructions. Following a single subtraction reaction, cDNA fragments from the forward and reverse reaction were cloned into pGEMT-vector (Promega, Heidelberg, Germany) and transformed in competent JM105 *Escherichia coli*. Plasmid DNA of 100 randomly selected clones from the forward reaction was sequenced by the cycle-sequencing method using dye terminators and the ABI 373 sequencer (Applied Biosystems, Weiterstadt, Germany).

Western Blot

Total protein extracts were isolated after homogenization of shock-frozen heart tissue in 2% sodium dodecyl sulfate (SDS), 50 mmol/L Tris (pH 6.8), 100 mmol/L DTT, 0.01%

Table 2. First and Second Antibodies Applied in this Study

First (F) or second (S) antibody, source	Concentration
ALCAM (CD166), Santa Cruz (N-21), F	1:400
BLCAM (CD22), Serotec (MCA1809), F	1:200
BLCAM (CD22), Santa Cruz (H-221), F	1:400
C-CAM1 (CD66), Santa Cruz (N-19), F	1:400
HCAM (CD44), Santa Cruz (DF 1485), F	1:400
HCAM (CD44), Santa Cruz (F-4), F	1:200
ICAM-1 (CD54), Santa Cruz (G-5), F	1:400
ICAM-2 (CD102), Santa Cruz (F-5), F	1:400
ICAM-3 (CD50), Oncogene (Ab-2), F	1:600
ICAM-3 (CD50), Santa Cruz (3.1), F	1:400
KALIG-1, Santa Cruz (C-20), F	1:400
NCAM(CD56), Novocastra (1B6), F	1:100
NCAM(CD56), Santa Cruz (123C3), F	1:400
PECAM-1 (CD31), Novocastra, F	1:100
VCAM-1 (CD106), Serotec (IE5), F	1:100
VCAM-1(CD106), Santa Cruz (C-19), F	1:400
Muscle-specific actin, Novocastra (HHF35), F	1:400
RUNX1 (AML1), Santa Cruz (N-20) (N terminus), F	1:400
RUNX1 (AML1), Santa Cruz (C-19) (C terminus), F	1:400
CREB-1, Santa Cruz (C-21), F	1:400
Hox D9, Santa Cruz (H-342), F	1:400
PAX 2/5/8, Santa Cruz (F-19), F	1:400
PAX 3/7, Santa Cruz, (C-20), F	1:400
rabbit-anti-mouse Ig, DAKO, S	1:2000
goat-anti-rabbit Ig, DAKO, S	1:2000
rabbit-anti-goat Ig, DAKO, S	1:2000

bromophenol blue, and separated on SDS-polyacrylamide gel electrophoresis (PAGE).[18] After blotting on nitrocellulose membrane, proteins were stained by Ponceau red. Blocking of membranes and incubations with the first and second antibody (2 hours each; Table 2) were carried out using 1X TBS/1X Tween 20/10% milk powder. Immunocomplexes were detected by the enhanced chemiluminescence system (Gibco, Heidelberg, Germany).

RNase Protection Assay

Total RNA of human heart was processed according to PharMingen's (San Diego, CA) RiboQuant protocol using anti-sense RNA as probe. Probes were generated using the following PCR primers: NCAM(CD56) UP: 5′-GAC GGC GGC TCC CCC ATC AGA-3′; NCAM(CD56) LP: 5′-ATG ACG ATG AGG ATG CCC ACG-3′; RUNX1(AML1) UP: 5′-TGC GCA CCG ACA GCC CCA ACT-3′; RUNX1(AML1) LP: 5′-GCT GTG TCT TCC TCC TGC ATC-3′; GAPDH UP: 5′-CAA CAG CGA CAC CCA CTC CTC-3′; GAPDH LP: 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′). PCR products were cloned into pGEM T-vector (Promega) and sequenced. Plasmid DNA was linearized with *Not*I RUNX1(AML1) and NCAM(CD56) or *Sal*I (GAPDH), labeled by *in vitro* transcription (PharMingen) with [$^{32P}\alpha$]UTP and separated by PAGE on a 6% acrylamide gel. Labeled RNA was extracted for 1 hour in 1 mol/L ammonium acetate at 60°C.

The predicted length of the transcripts were: NCAM(CD56), unprotected 360 bp/protected 283 bp; RUNX1(AML1), unprotected 592 bp/protected 527 bp; GAPDH, unprotected 233 bp/protected 156 bp.

Table 3. WT and Mutant (MUT) Oligonucleotides Used in EMSA

Oligonucleotide	Sequence
NCAM(CD56)-WT-sense	5′-TAA TAA AGA GAC CAC AGA TTT CAG AA-3′
NCAM(CD56)-WT-antisense	5′-TTC TGA AAT CTG TGG TCT CTT TAT TA-3′
NCAM(CD56)-MUT-sense	5′-TAA TAA AGA GAC GCC AGA TTT CAG AA-3′
NCAM(CD56)-MUT-antisense	5′-TTC TGA AAT CTG GCG TCT CTT TAT TA-3′
GMCSF-WT-sense	5′-GGC ATT TTG TGG TCA CCA TTA ATC-3′
GMCSF-WT-antisense	5′-GAT TAA TGG TGA CCA CAA AAT GCC-3′
GMCSF-WT-sense	5′-GGC ATT TTG GCG TCA CCA TTA ATC-3′
GMCSF-WT-antisense	5′-GAT TAA TGG TGA CGC CAA AAT GCC-3′

Immunohistochemistry

The anti-human-NCAM(CD56) monoclonal mouse antibodies 1B6 (Novocastra, Newcastle, UK) and 123C3 (Santa Cruz Biotechnology, Santa Cruz, CA) and the anti-rat-NCAM(CD56) rabbit polyclonal antibody H-300 (Santa Cruz) were used at 1:100 dilution in a four-step immunoperoxidase labeling for single antigens in formalin-fixed, paraffin-embedded sections (2-μm-thick).[19]

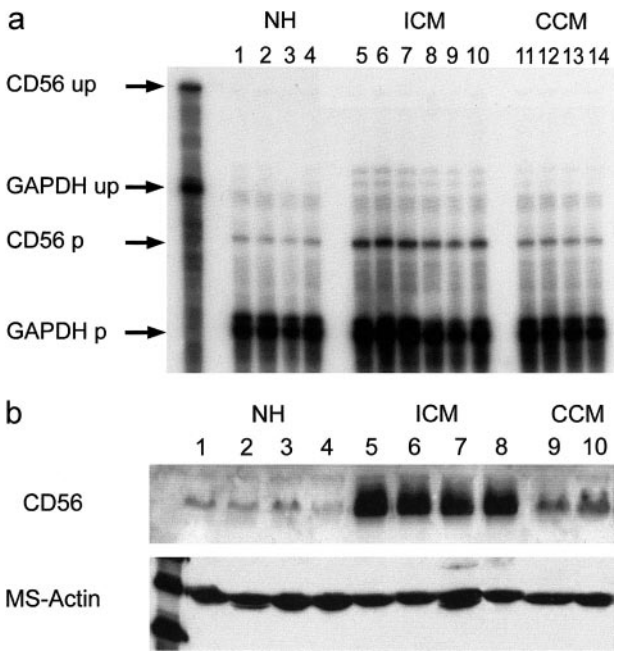
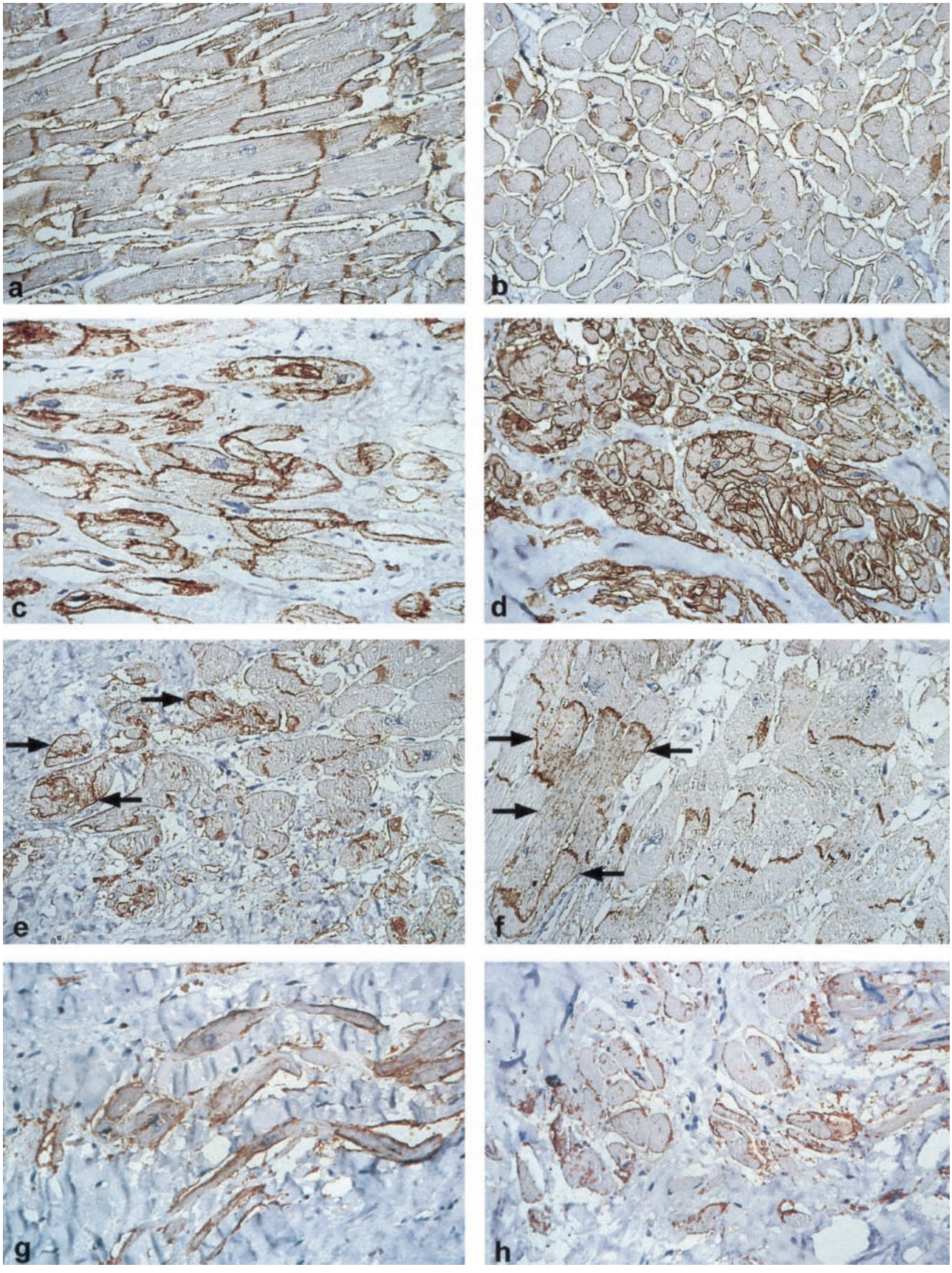


Figure 1. RNase Protection Assay (**a**) and Western blot (**b**) of normal human control hearts (**lanes 1** to **4** in **a** and **b**) and ICM hearts (**a**, **lanes 5** to **10**; **b**, **lanes 5** to **8**). Strong overexpression of NCAM(CD56) message and protein in ICM. Slight but significant NCAM(CD56) mRNA and protein overexpression in CCM (**a**, **lanes 11** to **14**; **b**, **lanes 9** to **10**). Equal loading of mRNA or protein, respectively, per lane checked by GAPDH (**a**) and muscle-specific actin Western blot (**b**). up, unprotected radioactive probe; p, protected radioactive probe.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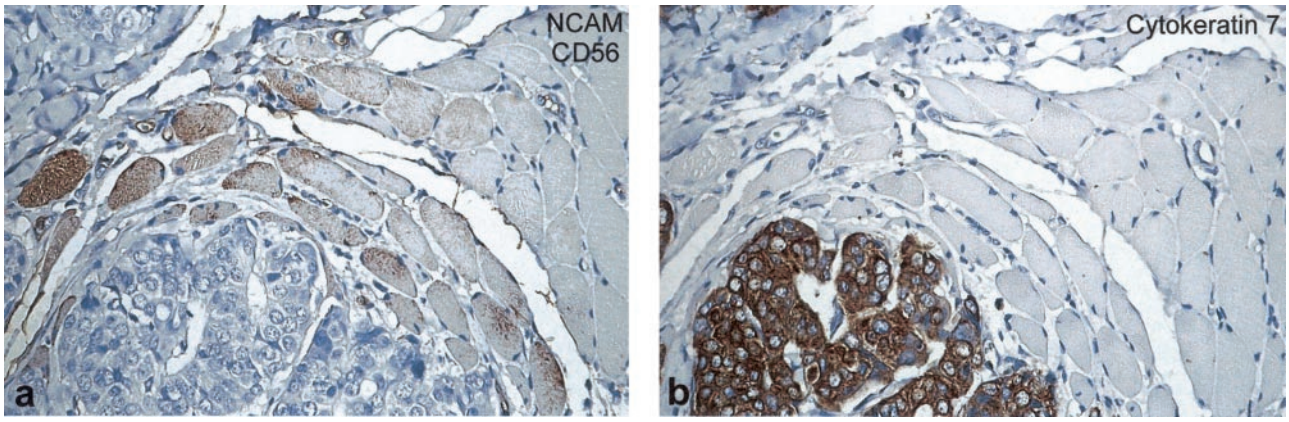


Figure 3. Immunohistochemical staining for NCAM(CD56) (**a**) and cytokeratin 7 (**b**) of a breast carcinoma infiltrating the musculus pectoralis major. Muscle fibers surrounded by or directly adjacent to carcinoma cells overexpress NCAM(CD56) protein. No NCAM(CD56) overexpression in muscle fibers at a distance from the carcinoma cells.

Electromobility Shift Assay

Jurkat cell nuclear extracts were isolated as described.[18] As probes, synthetic oligonucleotides containing a WT or mutant RUNX1(AML1) consensus sequence from the NCAM(CD56) and GMCSF promoter[20] (Table 3) were radioendlabeled with [γ^{32}P]ATP (Amersham Biosciences, Inc., Heidelberg, Germany). As controls, nonradioendlabeled mutant or WT consensus oligonucleotides were mixed with nuclear extracts either with or without anti-RUNX1(AML1) antibody, incubated with 1X binding buffer and 1 μg/μl poly(dI-dC) for 30 minutes on ice and analyzed on a 6% polyacrylamide gel in 0.25 TBE for 3 hours at room temperature. Autoradiography of vacuum-dried gels was carried out for 24 to 96 hours at −70°C.

Results

NCAM is Overexpressed in ICM

Among 100 sequenced clones from the PCR-subtract forward reaction, 7 clones (coding for *Myoglobulin*, *Myosin regulatory light chain*, *Myosin light chain*, *CD59*, *SLIM-1*, *actin*, *ubiquitin*) were identical to recently reported strong candidates for differentially expressed genes in cardiac hypertrophy.[21] In addition we isolated a 286-bp fragment homologous to the nucleotide sequence 1950–2236 of the 3′ end of NCAM(CD56). Since NCAM(CD56) had not been identified among the candidate "cardiac hypertrophy genes" in the previous extensive search by Hwang et al,[21] we concluded that NCAM(CD56) might be a candidate "ICM gene".

Strong overexpression of NCAM(CD56) mRNA occurred in all human ICM samples compared to normal hearts irrespective of the presence of previous myocardial infarction,[5,6] whereas CCM showed only a slight up-regulation (Figure 1a). By Western blot, all ICM samples strongly overexpressed the 125-kd NCAM(CD56) isoform, while normal heart samples expressed only traces of NCAM(CD56), and again CCM samples revealed slight but significant overexpression. (Figure 1b). By immunohistochemistry, NCAM(CD56) protein in normal hearts was detectable only at the disci intercalcares (Figure 2, a and b), while NCAM(CD56) in ICM was overexpressed on the whole cell membrane and in the cytoplasm (Figure 2, c and d). NCAM(CD56) overexpression was restricted to cardiomyocytes inside and adjacent to scars in ICM, while apparently normal and hypertrophic cardiomyocytes without contact to scars expressed NCAM(CD56) at control levels (Figure 2e). In CCM a focal and slight increase of NCAM(CD56) in the cytoplasma but not on the cell membrane was seen (Figure 2f), and either slight but significant, or no up-regulation was seen on cardiomyocytes inside or adjacent to formations of larger regions of interstitial fibrosis (Figure 2, g and h). The pattern of NCAM(CD56) overexpression in ICM is strikingly similar to the pattern observed in tumor-infiltrated skeletal muscle (Figure 3). Like in cardiomyocytes inside and directly adjacent to ischemic scars, NCAM(CD56) overexpression is strictly confined to skeletal muscle fibers surrounded by and in direct contact with carcinoma infiltrates.

NCAM(CD56) Overexpression in a Rat Model of ICM

To further investigate the role of NCAM(CD56) as a candidate "ICM gene", we analyzed its expression in well-established rat models of ICM and cardiac hypertrophy.[17] An identical pattern of NCAM(CD56) overexpression occurred in experimental ICM of rat hearts (Figure 4b) compared to sham-operated and spontaneously hypertensive rat hearts (Figure 4, a and c).

Figure 2. NCAM(CD56) expression in normal human heart at the disci intercalcares in longitudinal (**a**) and cross-sections (**b**). Overexpression and abnormal membrane and cytoplasmatic staining in ICM in longitudinal (**c**) and cross-sections (**d**). Restriction of NCAM(CD56) overexpression to cardiomyocytes inside and in contact with a scar (**arrows**) in ICM (**e**). Focal and slightly increased expression of NCAM(CD56) in the cytoplasma of cardiomyocytes (**f**) as well as in cardiomyocytes inside and adjacent to scars in longitudinal (**g**) and cross-sections (**h**) in CCM. Immunoperoxidase, ×200.

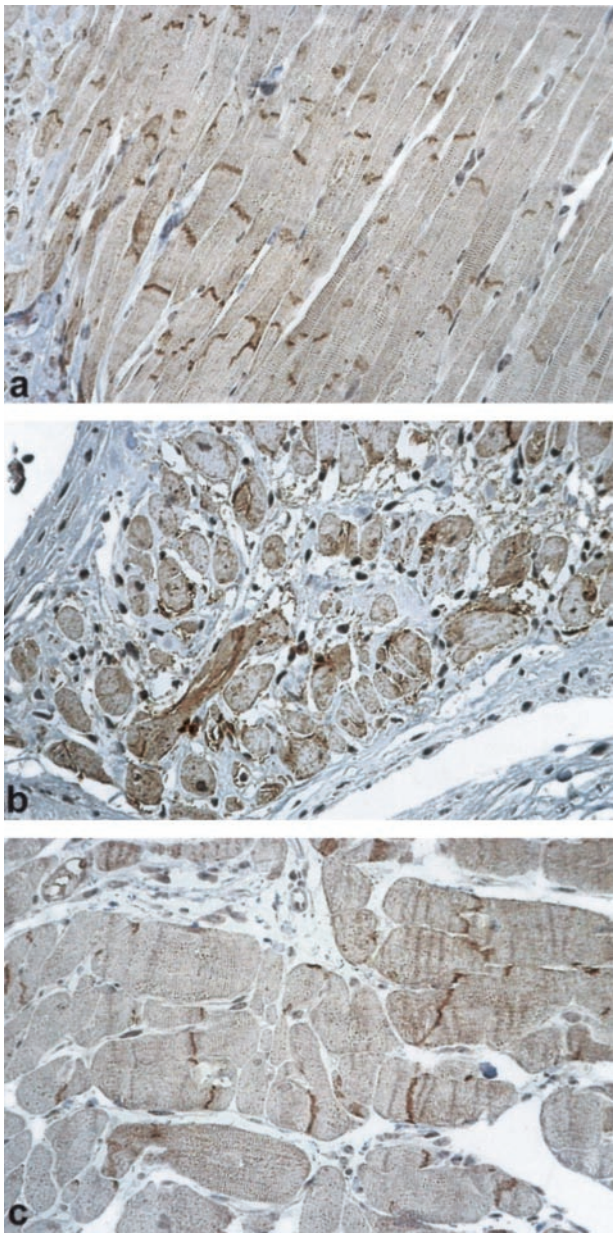


Figure 4. Immunohistochemical detection of NCAM(CD56) at the disci intercalcares in a normal rat heart (**a**). NCAM(CD56) overexpression on the whole cell membrane and in the cytoplasm of cardiomyocytes adjacent to a cardiac scar in rat heart with experimental ICM (**b**). Normal cardiac NCAM(CD56) expression in a spontaneously hypertensive rat (**c**). Immunoperoxidase, ×200.

NCAM(CD56) Is Not or Only Slightly Overexpressed in Heart Disorders Other than ICM

Tissue samples from patients suffering from heart diseases other than ICM (Figure 5a, c, e, g), including sarcoidosis (n = 2), myocarditis (n = 4) and HOCM (n = 2), were immunohistochemically stained for NCAM(CD56) (Figure 5b, d, f, h). Either slight (5b, sarcoidosis) or no recognizable overexpression (5 days, eosinophilic myocarditis; 5 hours, HOCM) of NCAM(CD56) was observed in these disease states. Furthermore, NCAM(CD56) was not overexpressed but even down-regulated on acutely infarcted cardiomyocytes (Figure 5f) as compared to normal cardiomyocytes. By contrast, viable cardiomyocytes within an ischemic scar adjacent to the acute infarct clearly showed up-regulated NCAM(CD56) expression (Figure 5f).

Other Cell Adhesion Molecules in ICM

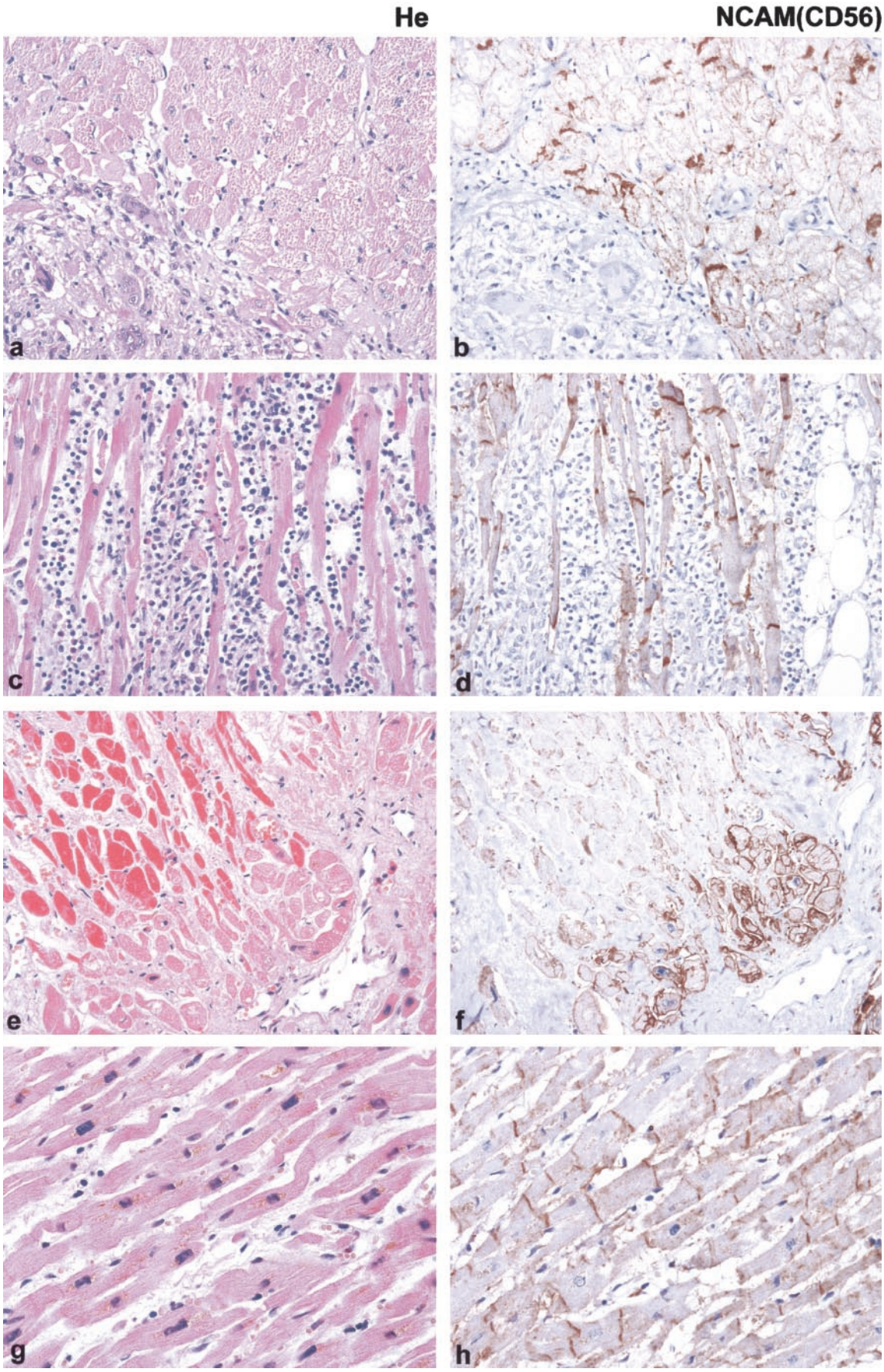
By Western blot, PECAM (CD31) revealed slight overexpression in ICM compared to controls (Figure 6). ICAM-1 (CD54) was expressed at high levels, and BL-CAM (CD22) (Figure 6), VCAM (CD106), and ALCAM (CD166) (not shown) were expressed at low levels in most cardiomyocytes of all samples. HCAM (CD44), ICAM-2 (CD102), ICAM-3 (CD50), KALIG, and C-CAM (CD66) proteins were undetectable (not shown).

RUNX1(AML1) is Overexpressed in ICM and Binds to the NCAM(CD56) Promoter

By Western blots of total protein extracts, the NCAM(CD56)-targeting transcription factors NF-kappa B p65 and p50, HOXD9, and PAX 2/5/8 were detected in ICM and normal hearts in similar amounts (Figure 7a). PAX3/7 protein was undetectable. By contrast, the 52-kd RUNX1(AML1) isoform, which is up-regulated in parallel to NCAM(CD56) in denervated mouse skeletal muscle,[22] and RUNX1(AML1) mRNA were overexpressed in all ICM specimens with only slight overexpression in CCM samples (Figure 7, b and c). In addition, a 38-kd band was detected in all heart extracts although down-regulated in ICM (Figure 7c).

To examine RUNX1(AML1) binding to the human NCAM(CD56) promoter, we performed electromobility shift assays (EMSAs) using Jurkat nuclear extracts as source of RUNX1(AML1) protein. RUNX1(AML1) formed a radioactive complex when mixed with radiolabeled WT NCAM(CD56) consensus probe (Figure 8). The specificity of the complex was checked severalfold: 1) complex formation did not occur with a radioactive mutant NCAM(CD56) probe; 2) the complex disappeared when non-radioendlabeled competitor NCAM(CD56) or GM-CSF consensus probes were added in excess; 3) a supershift of the DNA-protein complex occurred when anti-RUNX1(AML1) antibody was added to Jurkat nuclear extract mixed with radioendlabeled WT NCAM(CD56) consensus probe.

Figure 5. Various cardiac pathologies and associated NCAM(CD56) expression. Autopsy samples of heart tissue from patients suffering from sarcoidosis (**a** and **b**), eosinophilic myocarditis (**c** and **d**), acute heart infarction adjacent to an old ischemic scar (**e** and **f**) and HOCM (**g** and **h**). Either very slight or no up-regulation of NCAM(CD56) is seen on the cardiomyocytes adjacent to or within the inflammatory infiltrate in sarcoidosis, eosinophilic myocarditis, and HOCM. In a case of ICM (case 313/95, **e** and **f**) clear overexpression of NCAM(CD56) is detected in the cytoplasma and on the whole cell membrane of viable cardiomyocates within or adjacent to scars (lower right half) whereas no NCAM(CD56) expression is detectable on non-viable, hypereosinophilic cardiomyocytes in a region of acute cardiac infarct (upper left half). HE, Immunoperoxidase ×200.


He
NCAM(CD56)
a
b
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d
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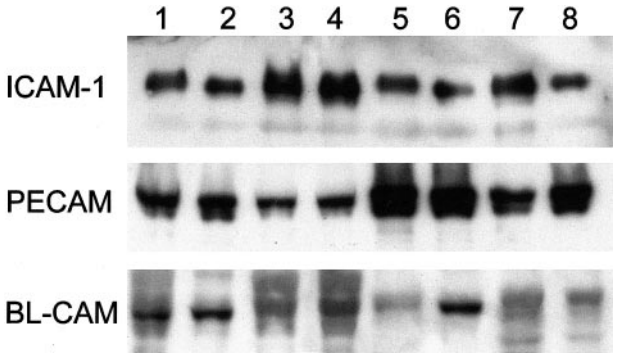

Figure 6. Western blots of normal human hearts (**lanes 1** to **4**) and ICM (**lanes 5** to **8**) with antibodies against ICAM-1 (CD54), PECAM (CD31), and BL-CAM (CD22). Slight increase only of PECAM in ICM compared to control.

Discussion

NCAM(CD56) is a member of the immunoglobulin superfamily, mediating intercellular adhesion in the nervous system and skeletal muscle.[23–25] NCAM(CD56) expression was also found at the disci intercalcares in normal human hearts and cardiac allografts.[26,27] A correlation between NCAM(CD56) expression levels and heart pathology has not been reported. Therefore, it is our main finding that strong overexpression of NCAM(CD56) is consistently associated with ICM (Figures 1, 2, 4 and 5). Strong NCAM(CD56) protein overexpression was restricted to cardiomyocytes associated with ischemia-induced scars and was qualitatively different from expression in normal hearts since it involved the whole cardiomyocyte cell membrane and cytoplasm (Figure 2, c and d). This NCAM(CD56) expression pattern appears characteristic for cardiomyocytes within or adjacent to scars in ICM, while at most slight overexpression was found in hearts from patients with CCM, HOCM, sarcoidosis, and myocarditis (Figures 2 and 5). The relationship of the findings to ischemia is supported by identical observations in a rat model of ICM[17] (Figure 4b) but not in hearts of spontaneously hypertensive rats (Figures 4, a and c). The findings imply that NCAM(CD56) overexpression is neither a marker for cardiac hypertrophy nor for contractile insufficiency.

The second new finding is the identification of RUNX1(AML1) as a transcription factor that is up-regulated in parallel to NCAM(CD56) overexpression in ICM (Figure 7). Overexpression of the 52-kd RUNX1(AML1) isoform in parallel to NCAM(CD56) in ICM and the demonstration that RUNX1(AML1) has a binding site within the NCAM(CD56) promoter suggest that RUNX1(AML1) plays a role in NCAM(CD56) regulation. This hypothesis is supported by two additional findings. First, other transcription factors with potential binding sites within the NCAM(CD56) promoter (like NF-kappa B[28]) did not exhibit differential expression in ICM as compared to controls (Figure 6a). The findings do not exclude, however, that constitutively expressed NF-kappa B, HOX, PAX, or other transcription factors contribute to ICM-related NCAM(CD56) expression. Second, hyperexpression of the 52-kd RUNX1(AML1) isoform in ICM was accompanied by down-regulation of a 38-kd band (Figure 6c) that

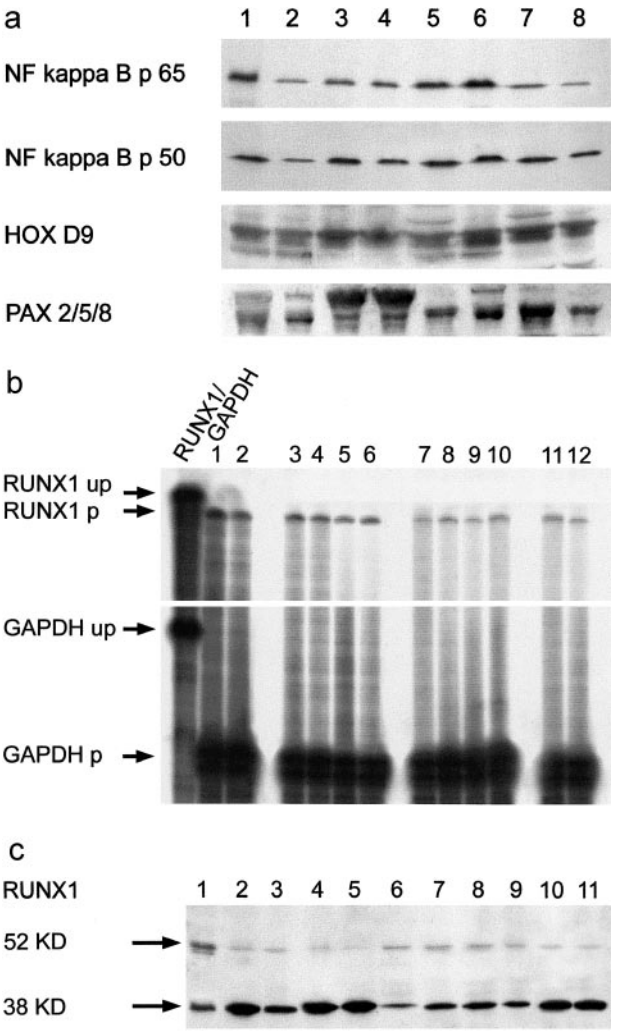

Figure 7. Detection of NCAM(CD56)-related transcription factors NF-kappa B, HOXD9, PAX (**a**), and RUNX1(AML1) (**b** and **c**) in normal and ICM hearts. **a:** Similar amounts of NF-kappa B p65 and p50, HOXD9, and PAX 2/5/8 in normal (**lanes 1** to **4**) and ICM hearts (**lanes 5** to **8**) (Western blots). **b:** Overexpression of RUNX1(AML1) mRNA in ICM (**lanes 3** to **6**) compared to normal human hearts (**lanes 7** to **10**) and slight overexpression in CCM (**lanes 11** to **12**). Jurkat-derived (**lane 1**) and TE671 rhabdomyosarcoma-derived (**lane 2**) control mRNAs (RNase protection assays). **c:** Overexpression of the 52-kd RUNX1(AML1) isoform[30] in ICM (**lanes 6** to **9**) compared to normal human hearts (**lanes 2** to **5**) and again either very slight or no overexpression in CCM (**lanes 10** to **11**) (Western blots). Jurkat cell extract (**lane 1**, positive control). Strong expression of an additional 38-kd band in all samples, with down-regulation in ICM (**lanes 6** to **9**) and CCM (**lanes 10** to **11**). up, unprotected radioactive probe; p, protected radioactive probe

reacted with an antibody against the N terminus of RUNX1(AML1). This band might correspond to the 38-kd RUNX1(AML1) delta N isoform[29] that has a known dominant negative function. Thus, the findings suggest that the active RUNX1(AML1) isoform is up-regulated while a dominant negative isoform is down-regulated in ICM. Since there are other RUNX1(AML1) isoforms with dominant negative effects,[30–32] it appears warranted to analyze ICM hearts for the whole spectrum of RUNX1(AML1) isoforms.

The functional significance of NCAM(CD56) overexpression in ICM is unknown, as are the trigger(s) underlying RUNX1(AML1) up-regulation. One possible indica-

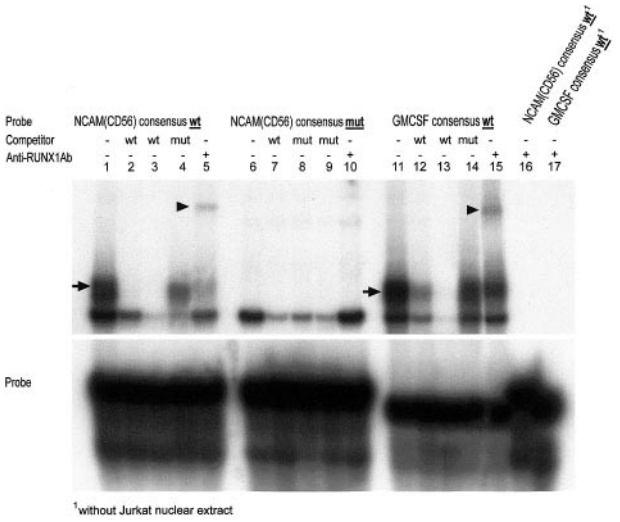

Figure 8. Detection of specific RUNX1(AML1) protein binding within the human NCAM(CD56) promoter by electromobility shift assay (EMSA). Radioactive DNA-protein complex formation by mixing RUNX1(AML1) protein-expressing Jurkat cell extract with [γ-^{32}P]ATP radioendlabeled WT NCAM(CD56) consensus probe (**lanes 1**, **4**, and **5**, **arrow**) and as control with [γ-^{32}P]ATP radioendlabeled WT GMCSF consensus probe (**lanes 11**, **12**, **14** and **15**, **arrow**) but not with [γ^{32}P]ATP radioendlabeled mutant (mt) NCAM(CD56) probe (**lanes 6** to **10**). Supershifts of the specific complexes by adding anti-RUNX1(AML1) mAb (**lanes 5** and **15**, **arrowheads**). No complex formation between the anti-RUNX1(AML1) antibody and radioendlabeled WT NCAM(CD56) (**lane 16**) or WT GMCSF (**lane 17**) consensus probes in the absence of RUNX1(AML1) (ie, Jurkat nuclear extract).

tion of the trigger(s) is the restriction of NCAM(CD56) overexpression to the vicinity of ischemia-induced scars. This expression pattern is strikingly similar to that encountered in tumor-infiltrated human skeletal muscle (Figure 3). Skeletal muscle fibers separated from each other by tumor cells up-regulate NCAM(CD56), while adjacent tumor-free muscle fibers do not. Therefore, we suggest that loss of cell-cell interaction might trigger NCAM(CD56) gene expression in cardiac and skeletal muscle, respectively. This hypothesis is analogous to the findings of Saffitz et al,[33,34] who showed that down-regulation of the gap junction protein connexin43 is due to loss of cell-cell interaction. Since this effect is mediated by stress-induced activation of c-JUN N-terminal kinase,[35] it will be interesting to analyze NCAM expression in relation to c-JUN kinase activity.

According to this hypothesis, concomitant up-regulation of NCAM(CD56) and RUNX1(AML1) is not only a feature of ICM (Figures 2, 3, and 7) and tumor-infiltrated skeletal muscle (Figure 3) but also of muscle after denervation.[22] Interestingly, identical triggers target AChR-γ-subunit gene expression in skeletal muscle.[19,26,36,37] Thus, as a common theme, "communication failures" by either neuromuscular blockade or disruption of (cardio)-myocyte-(cardio)myocyte contacts stereotypically are associated with increased transcription of the NCAM(CD56), RUNX1(AML1), and AChR-γ-subunit genes. Therefore, it will be interesting to determine whether denervation-related genes in addition to RUNX1(AML1) and NCAM(CD56) play a role in ICM.

In summary, we identified NCAM(CD56) hyperexpression as a local response of cardiomyocytes to scar formation in ICM. This response is probably regulated by various RUNX1(AML1) isoforms that we suspect to act as positive or dominant-negative effectors targeting the NCAM(CD56) promoter.

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