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Role of Desmin Filaments in Chicken Cardiac Myofibrillogenesis

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Abstract Desmin filaments are muscle-specific intermediate filaments located at the periphery of the Z-discs, and they have been postulated to play a critical role in the lateral registration of myofibrils. Previous studies suggest that intermediate filaments may be involved in titin assembly during the early stages of myofibrillogenesis. In order to investigate the putative function of desmin filaments in myofibrillogenesis, rabbit anti-desmin antibodies were introduced into cultured cardiomyocytes by electroporation to perturb the normal function of desmin filaments. Changes in the assembly of several sarcomeric proteins were examined by immunofluorescence. In cardiomyocytes incorporated with normal rabbit serum, staining for α-actinin and muscle actin displayed the typical Z-line and I-band patterns, respectively, while staining for titin with monoclonal anti-titin A12 antibody, which labels a titin epitope at the A-I junction, showed the periodic doublet staining pattern. Staining for C-protein gave an amorphous pattern in early cultures and identified A-band doublets in older cultures. In contrast, in cardiomyocytes incorporated with anti-desmin antibodies, α-actinin was found in disoriented Z-discs and the myofibrils became fragmented, forming mini-sarcomeres. In addition, titin was not organized into the typical A-band doublet, but appeared to be aggregated. Muscle actin staining was especially weak and appeared in tiny clusters. Moreover, in all ages of cardiomyocytes tested, C-protein remained in the disassembled form. The present data suggest the essential role of desmin in myofibril assembly. J. Cell. Biochem. 77:635–644, 2000.

Key words: cardiomyocyte; desmin antibody perturbation; titin; C-protein; actin

Expression of desmin starts in the early myoblast stage of embryonic skeletal and cardiac muscle development [Gard and Lazarides, 1980]. Desmin filaments are first organized into a filamentous network, and then redistribute to the intermyofibrillar space and encircle the periphery of the Z-bands [Bennet et al., 1979; Gard and Lazarides, 1980; Fuselar and Shay, 1982; Danto and Fischman, 1984]. They are therefore suggested to keep adjacent myofibrils in registration [Lazarides and Hubbard, 1976; Gard and Lazarides, 1982].

In contrast, studies on embryonic skeletal muscle and cultured cardiomyocytes indicate that the registration of myofibrils occurs prior to the reorganization of desmin filaments to the Z-bands, suggesting that desmin filaments are not involved in the lateral registration of myofibrils [Tokuyasu et al., 1985; Fuseler and Shay, 1982; Bennett et al., 1979]. Transfection of desmin antisense RNA into C_2C_{12} myoblasts blocks the formation of myotubes and interferes with the expression of MyoD and myogenin, indicating that desmin is involved in gene regulation [Li et al., 1994]. In mouse embryonic stem cells, destruction of the desmin gene by homologous recombination results in failure of differentiation in three muscle tissues [Weitzer et al., 1995]. These observations demonstrate that desmin plays an important role in muscle development [Ingber, 1993; Li et al., 1994; Weitzer et al., 1995]. However, Schultheiss et al. [1991] showed that introduction of a truncated desmin gene into postmitotic myoblasts did not affect the normal development and

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alignment of myofibrils, which again raises the question of the function of desmin. In a study using desmin gene-knockout mice, Milner et al. [1996] reported that, in prenatal animals, myofibril assembly of striated muscle proceeded normally, but that skeletal and cardiac myofibrils in postnatal animals became disorganized and reduced in size. Similar results were obtained by Li et al. [1997] and Thornell et al. [1997], who concluded that the early phase of myofibrillogenesis does not require desmin filaments, but that desmin filaments may be involved in the later stabilization and repair of myofibrils. The exact role of desmin filaments in the later phase of myofibrillogenesis remains obscure.

An immunoelectron microscopic study on cultured human skeletal myotubes demonstrated the association of titin aggregates with intermediate filaments [van der Ven et al., 1993] and that this intermediate filament-associated titin was in close contact with a subsarcolemmally located stress fiber-like structure. These authors suggested that these intermediate filaments may be related to the intracellular transport of titin to the stress fiber-like structure. Whether these intermediate filaments consist of desmin is not known. The present work aimed at investigating the putative function of desmin by incorporation of rabbit anti-desmin antiserum into developing cardiomyocytes to perturb the normal function of desmin and to examine the effect on myofibril assembly with special attention to titin, α-actinin, C-protein, and actin.

MATERIALS AND METHODS

Cell Culture

Primary cultures of chicken embryonic cardiomyocytes were prepared according to the method of Lin et al. [1989]. Ventricular tissues were removed from the hearts of 8-day-old chicken embryos, cut into pieces, and dissociated with 0.05% trypsin-EDTA in Ca^{2+}, Mg^{2+}-free Hank's buffer. The dispersed cells were collected by centrifugation, resuspended, and plated on collagen-coated coverslips at an optimal culture density of 2–3.5 $\times 10^5$ cells/ml. The culture medium was minimal essential medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum, 2 mg/ml of glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin for the first day of culture; this was then changed to glutamine-free medium.

Electroporation

Before electroporation, day 3 cultures were washed twice with electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, pH 7). The coverslips and attached cells were transferred to a Gibco pulser cuvette containing 1 ml of antibody solution [200 μl of rabbit anti-desmin antiserum (Sigma, St. Louis, MO) plus 800 μl of electroporation buffer], and then placed on ice for 5 min. For electropermeabilization, a cell porator (BRL, Gaithersburg, MD; settings 750 V/cm and 60 μF capacitance) was used. Control cells underwent electroporation in the presence of normal rabbit serum (Sigma). Immediately after electroporation, the cells were left in the cuvette on ice for another 15 min, washed twice with culture medium, and cultured in fresh medium for 2 h at 37°C in a CO_2 incubator before immunostaining. The incorporated normal rabbit IgG or anti-desmin antibody was visualized by subsequent staining with FITC-conjugated goat anti-rabbit IgG.

Immunofluorescent Staining

The cell cultures were fixed with 10% formalin in phosphate-buffered saline (PBS) for 10 min, and nonspecific binding sites were blocked at room temperature for 15 min with PBS containing 5% nonfat milk and 0.1% Triton X-100. The cells were then washed with PBS and incubated with primary antibodies for 1 h at 37°C. Rabbit polyclonal anti-desmin antiserum (Sigma), rat anti-skeletal α-actinin antiserum [Wang et al., 1998], mouse monoclonal anti-skeletal titin A12 antibody [Wang et al., 1998], mouse monoclonal anti-muscle fast C protein ascites (MF1, Developmental Studies Hybridoma Bank, Iowa City, IA), and FITC-phalloidin (Sigma) were used to study the assembly of myofibrillar proteins in cardiomyocytes. An appropriate combination of two primary antibodies was used in double-labeling studies. Second antibody treatment consisted of incubation of the cells for 1 h at 37°C with the appropriate detection agent (FITC-conjugated goat anti-rabbit IgG antibody, FITC-conjugated goat anti mouse γ-chain-specific antibody, Texas-red goat anti-rat IgG, Texas-red goat anti-rabbit IgG, or biotinylated horse anti-mouse

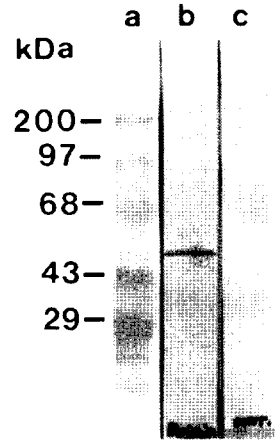

Fig. 1. Western blot analysis of rabbit anti-desmin antiserum. **Lane a,** prestained molecular weight markers; **lane b,** immunoblot of embryonic cardiac muscle proteins using anti-desmin antiserum; **lane c,** control. Omission of the primary antibody shows a clear background.

IgG plus avidin-Texas red; Vector, Burlingame, CA), followed by rinsing with PBS and mounting in 0.1 M phosphate buffer, pH 8, containing 2% n-propyl gallate and 60% glycerol. Observations were made using a Reichert Polyvar 2 microscope (Leica, Wien, Austria) equipped with epifluorescence illumination.

RESULTS

Antibody Specificity

The specificity of rabbit anti-desmin antiserum used in this study was examined by Western blot analysis. Using chicken cardiac muscle lysates, the antibody was shown to react only with desmin (Fig. 1).

Electroporation Control

In order to understand the function of desmin filaments in the assembly of α-actinin, titin, C-protein, and actin during the assembly of sarcomeres, we introduced anti-desmin antiserum into cultured cardiomyocytes to interfere with the normal function of desmin filaments, and examined the distribution patterns of several contractile proteins.

For the control group, normal rabbit serum was used in the electroporation buffer. As shown in Figure 2, in these control cells, α-actinin staining was seen in the periodic Z-bands (Fig. 2A,B), titin staining at the A-I junctions of a sarcomere, which appears as a doublet in long sarcomeres (Fig. 2C,D), and actin staining in the typical I bands (Fig. 2E,F). The data indicate that the electroporation condition did not affect the normal process of myofibril assembly.

α-Actinin and Titin Staining

Immunostaining for rabbit IgG in cells that had been incorporated with rabbit anti-desmin antibody appeared as amorphous, filamentous, or fibrillar patterns. This indicated that incorporated anti-desmin antibody specifically bound to desmin filaments. In contrast to the nonincorporated control cells, these cells showed significant changes in the distribution of contractile proteins, with α-actinin being found on the Z-line of short disorganized sarcomeres (cell 1 in Fig. 3A,B), while titin formed random aggregates and did not assemble into the typical A-band doublets (cell 1 in Fig. 3C,D), as seen in nearby nonincorporated cells (cell 2 in Fig. 3C,D).

C-Protein Staining

Previous biochemical studies confirmed the association of titin and C-protein [Koretz et al., 1993; Furst et al., 1992]. Because of the disturbance of titin assembly seen after anti-desmin antibody incorporation, we next examined the effect on C-protein staining. The distribution of C-protein in cultured cardiomyocytes at different stages changed from amorphous on day 2 (Fig. 4A,B) to a mature A-band doublet pattern on day 3 (Fig. 4C–F). Normally, C-protein took up its mature position at an earlier stage than the registration of α-actinin to the Z-line, as shown in Figure 4C,D. In the majority of normal rabbit IgG-incorporated cardiomyocytes on days 3–5 culture, C-protein was assembled into A-band doublets (Fig. 5A,B). However, in cardiomyocytes incorporated with anti-desmin antiserum, regardless of culture age (days 2–5), C-protein displayed only filamentous staining, an immature pattern for C-protein (Fig. 5C–H), whereas in normal nonincorporated cells, C-protein appeared in the A-band doublet pattern (Fig. 5C,D,G,H).

Actin Staining

In electroporation control cells, FITC-phallotoxin, which binds to polymerized actin filaments, labeled the I-band of mature myofibrils or premyofibrils (shown as continuous staining) (Fig. 2E,F), whereas in cardiomyo-

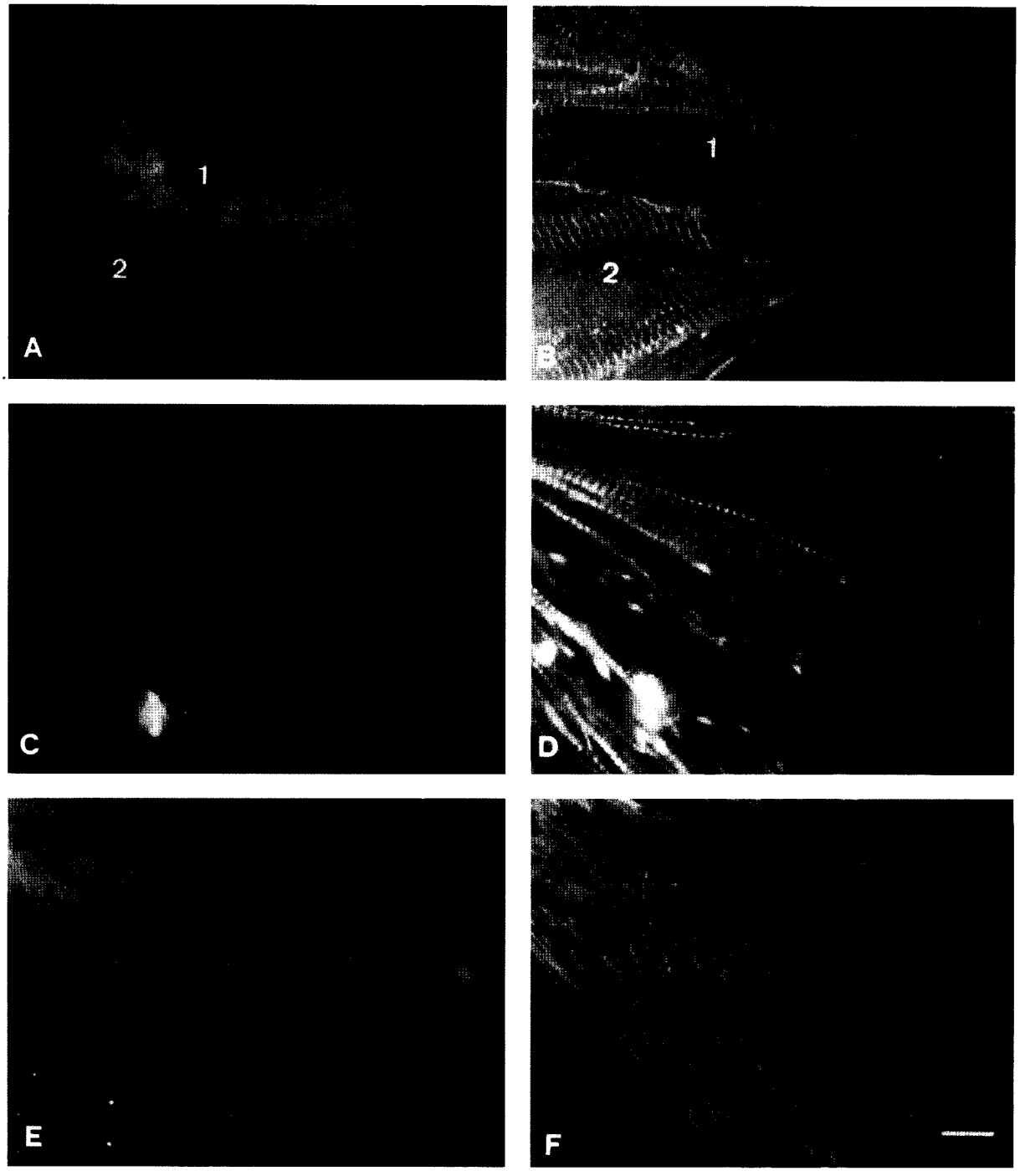

Fig. 2. Distribution of α-actinin, titin, and act n in day 3 cardiomyocytes treated with control rabbit serum. The cells were double-immunostained for rabbit IgG (**A**, **C**, **E**) and α-actinin (**B**), titin (**D**), or FITC-phallotoxin (**F**). The typical arrangement and staining patterns for α-actini n (Z-bands), titin (titin doublet in the A-band), and actin (I-bands) can be seen in both normal rabbit IgG-incorporate d cells (cell 1) and non-incorporated cells (cell 2). Scale bar, 10 μm.

cytes showing anti-desmin antiserum incorporation, only weak punctate staining was detected (Fig. 6A,B). Monoclonal anti-muscle actin ascites (clone AC40, Sigma), which reacts with both G-actin and F-actin, showed irregular plaques in the cytoplasm (Fig. 6C,D), suggesting abnormal assembly of actin filaments.

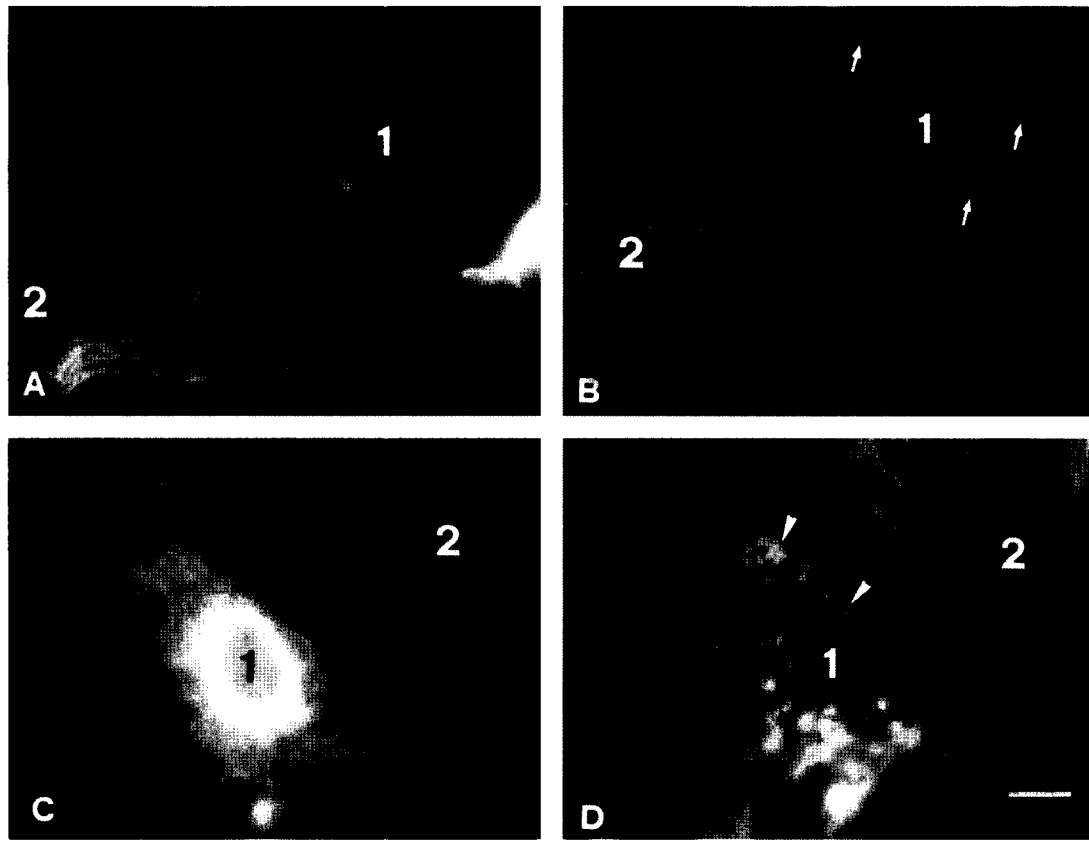

Fig. 3. Effect of rabbit anti-desmin antiserum incorporation on the assembly of α-actinin and titin. Day 3 cardiomyocytes were incorporated with rabbit anti-desmin antibody and double-stained for rabbit IgG (**A, C**) and either α-actinin (**B**) or titin (**D**). In cells with incorporated antibodies (cell 1 in A), α-actinin is not aligned in a linear array (cell 1), as is the case in nonincorporated cells (cell 2 in A). Myofibrils appear fragmented (arrows in B). Titin appears in clusters or aggregates in antibody-incorporated cells (arrowheads in D), whereas in cells without antibody incorporation (cell 2 in C), titin is arranged in a periodic pattern (cell 2 in A–D). Scale bar, 10 μm.

DISCUSSION

The elaborate organization of desmin has been suggested to be important in maintaining the structural integrity and function of cardiac muscle [Nag and Haffaker, 1998]. Transfection of a truncated desmin construct into C_2C_{12} muscle cells showed disruption of the endogenous desmin filament [Raats et al., 1996]. However, the mechanism by which truncated desmin affects myofibril formation remains unclear. To gain further insight into the function of desmin filaments in the assembly of myofibrillar proteins, we introduced anti-desmin antibody into developing cardiomyocytes by electroporation and examined the distribution of myofibrillar proteins. We demonstrated that the incorporated anti-desmin antibody had a profound effect on myofibrillar structure in developing cardiomyocytes at days 2–5 of culture, and that several contractile proteins, including titin, C-protein, and actin, failed to assemble into the sarcomeric structure.

Titin, an elastic protein in striated muscle, extends from the M-line to the Z-line region [Furst et al., 1988]. The Z-repeat region of titin is responsible for the control of Z-disk assembly [Peckham et al., 1997], and the A-band domain of titin is involved in the ordered assembly of thick filaments [Labeit et al., 1992]. As shown by an immunoelectron microscopic study, titin forms spots in the cytoplasm of embryonic cardiac muscle cells and cultured skeletal myoblasts [van der Ven et al., 1993; Fulton and Alftine, 1997; Tokuyasu and Maher, 1987a]. In cultured human skeletal myoblasts, titin aggregates are tightly associated with intermediate filaments of unknown type and are targeted to a stress fiber-like structure through intermediate filaments [van der Ven et al., 1993; 1996]. Perturbation of desmin filaments by anti-desmin electroporation

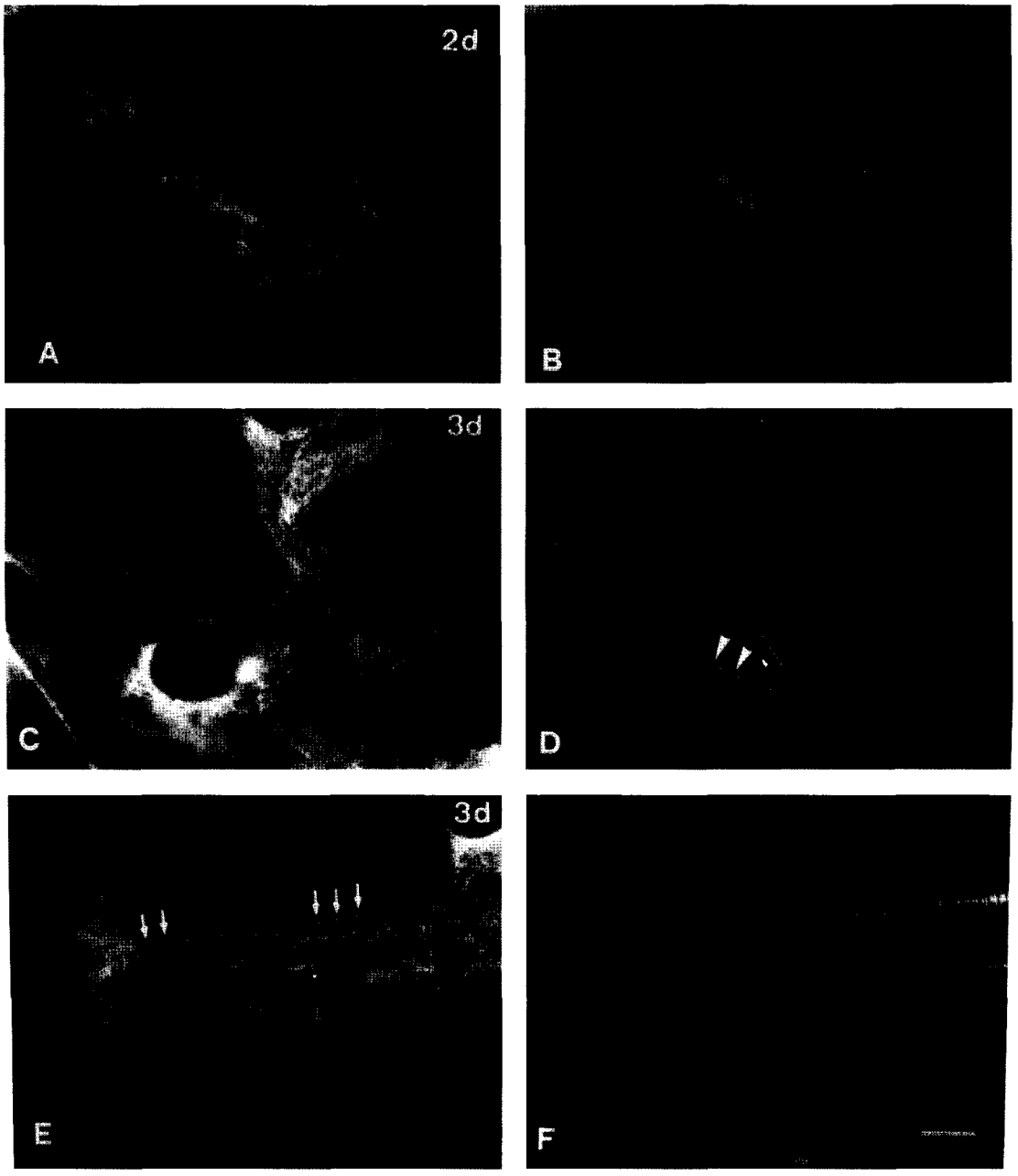

Fig. 4. C-protein staining patterns during normal cardiomyocyte development. Day 2 (A, B; 2d) and day 3 cells (C–F; 3d) were double-stained for desmin (**A, C, E**) and C-protein (**B, D, F**). C-protein is first seen as an amorphous pattern (B, D), and gradually becomes organized into doublets in the A-band (arrowheads in D). Desmin first appears as a filamentous network (A, C), and then changes to a periodic arrangement around the Z-lines (arrows in E). Scale bar, 10 μm.

resulted in the formation of titin aggregates, suggesting the involvement of desmin filaments in titin transport and assembly. These data suggest that the titin molecules were not correctly integrated into the sarcomere, which may result from loss of guidance by desmin filaments. In the cardiomyopathic myocardium, the relative amount of desmin is decreased and irregularly distributed [Kawaguchi et al., 1995] and, in addition, loss of cross-striation of α-actinin and titin is frequently observed. This observation supports the idea that the abnormality of desmin distribution is related to defective organization of α-actinin and titin.

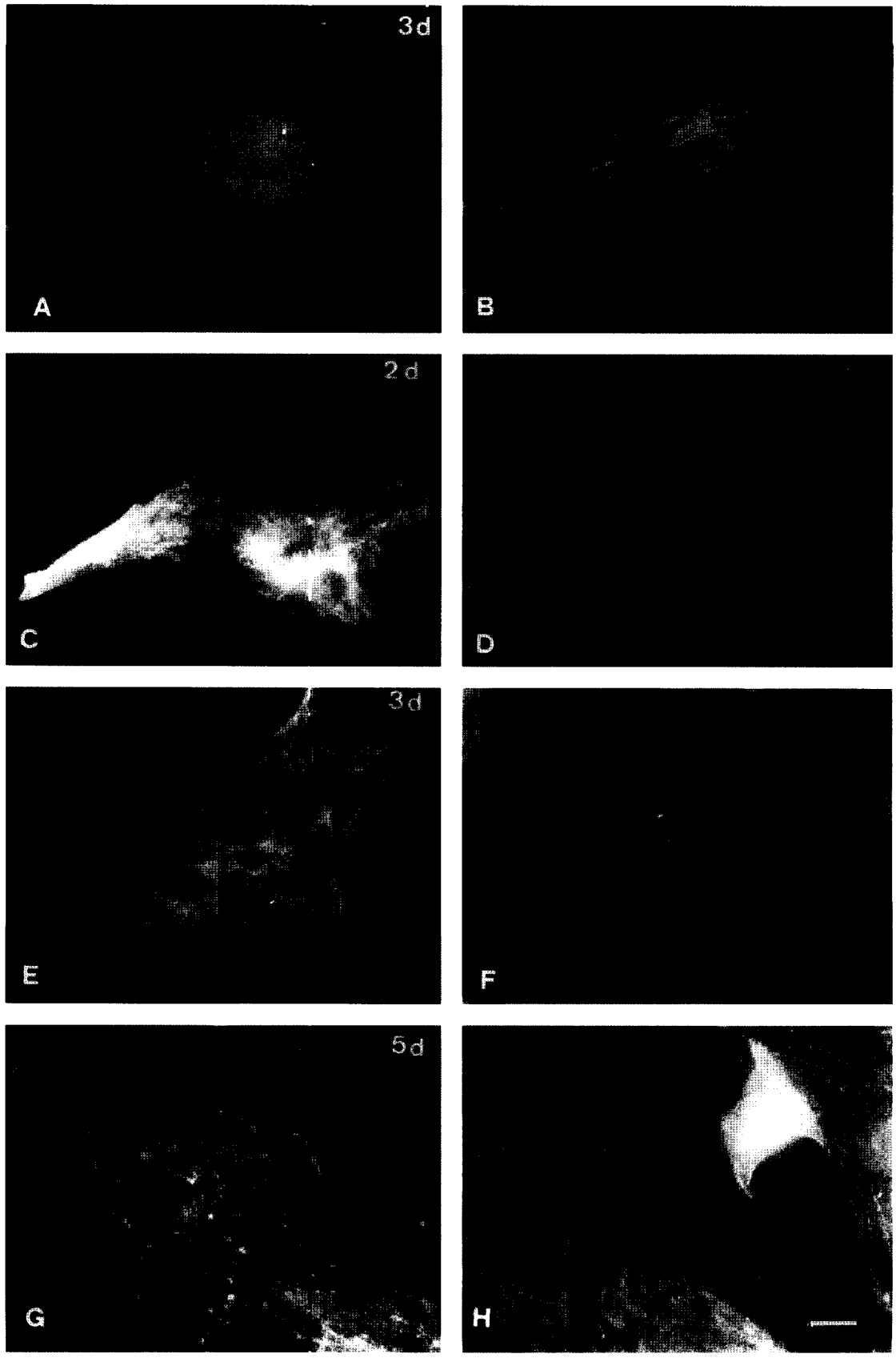

Fig. 5. Effect of anti-desmin antiserum incorporation on the distribution of C-protein in cardiomyocytes of various stages. A, B: Day 3 cells incorporated with control rabbit serum and double-stained for incorporated rabbit IgG (**A**) and C-protein (**B**), respectively. In these electroporation control cells, C-protein is organized into doublets in the A-band. Anti-desmin antiserum-incorporated cells from 2–5-day cultures (indicated as 2d, 3d, and 5d) were double-stained for incorporated rabbit IgG (**C, E, G**) and C-protein (**D, F, H**). C-protein fails to assemble into doublets in the A-band in anti-desmin-incorporated cardiomyocytes from day 2–5 cultures. In nearby nonincorporated cells, C-protein is assembled in typical doublets in the A-band (D, H). Scale bar, 10 μm.

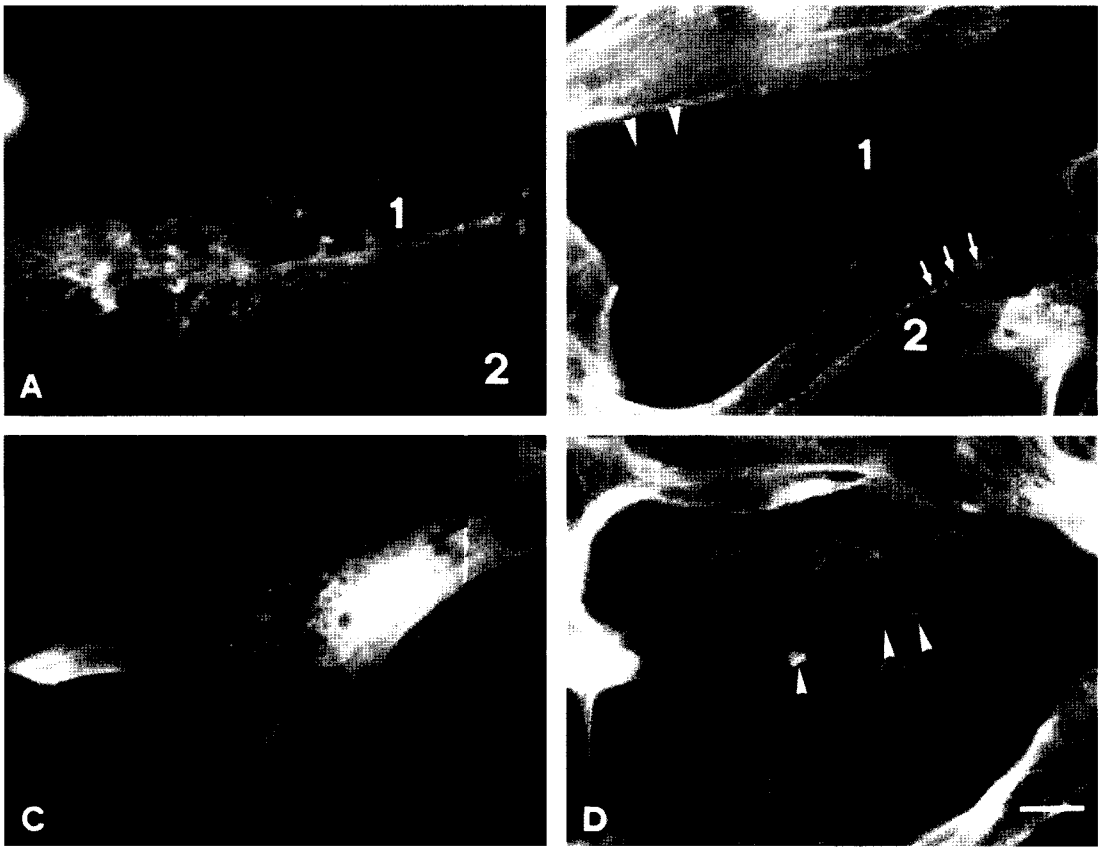

Fig. 6. Effect of anti-desmin antiserum incorporation on actin distribution. Antibody-incorporated cardiomyocytes (day 3) were double-labeled for rabbit IgG (**A, C**) and actin (**B**, FITC-phallotoxin; **D**, monoclonal anti-muscle actin). Using FITC-phallotoxin labeling (B), actin staining is very weak (arrowheads in cell 1), whereas anti-actin staining (arrowheads in D) shows irregular plaques in antibody-incorporated cells. Nearby nonincorporated cells show a strong I-band pattern (B, arrows in cell 2) or stress fiber-like staining (D). Scale bar, 10 μm.

At the onset of myofibrillogenesis, Z-bodies are formed as punctate concentrations of α-actinin [Tokuyasu and Maher, 1987b; Sanger et al., 1984], and this is followed by anchorage of the N-terminus of the titin molecule into the Z-body [Turnaciogly et al., 1997]. The subsequent alignment and fusion of Z-bodies of adjacent premyofibrils result in the formation of Z bands [Dabiri et al., 1997; Turnaciogly et al., 1997; Peckham et al., 1997; Sorimachi et al., 1997; Nag and Lee, 1997]. Incorporation of anti-desmin antibodies may block the interactions between desmin and α-actinin and α-actinin and titin, which would explain the formation of titin aggregates and the fragmentation of myofibrils. The binding between desmin and α-actinin may be important in the fusion of premyofibrils, since desmin filaments are suggested to link together adjacent myofibrils. The present finding that the growth in diameter of myofibrils was inhibited in antibody-incorporated cardiomyocytes is consistent with the above hypothetical role for desmin filaments.

C-protein is a component of the thick filaments and may regulate the actin-myosin interaction [Hartzell and Sale, 1985]. Since C-protein binds to myosin and titin, it has been suggested to play a role in sarcomere formation [Winegrad, 1999] and to prevent the stretching and extension of titin filaments in the A-band [Koretz et al., 1993]. Evidence for biochemical binding between titin and C-protein was obtained in previous studies [Koretz et al., 1993; Freiburg and Gautel, 1996; Gilbert et al., 1999; Winegrad, 1999]. In this study, C-protein was diffusely distributed in the cytoplasm of day 2 control cardiomyocytes, and then gradually became localized in the A-band on days 3–5, as reported by Koshida et al. [1995]. In anti-desmin antibody-incorporated cardiomyocytes, regardless of culture age, the staining of

C-protein consistently appeared as an amorphous pattern. The failure of assembly of C-protein into the sarcomere may be due to the aberrant distribution of titin (aggregate formation), which then disturbed the normal interaction between titin and C-protein.

There is biochemical evidence for the binding of titin to F-actin [Jin, 1995], C-protein to titin, and C-protein to actin [Freiburg and Gautel, 1996; Moos et al., 1978; Trombitas and Granzier, 1997; Yamamoto, 1986; Koretz et al., 1993; Soteriou et al., 1993]. The class I motif of titin binds to both myosin and actin in a solid-phase binding assay [Jin, 1995]. Trombitas and Granzier [1997] reported that titin is attached to the actin filaments, since removal of actin filaments by gelsolin causes the titin filaments to contract. An in vitro cosedimentation assay also confirmed that actin interacts with Z-band titin [Linke et al., 1997]. These interactions are essential in stabilizing the I-band structure. In anti-desmin-incorporated cardiomyocytes, titin was not assembled into sarcomeres. Disassembly of titin may interfere with its interaction with C-protein and F-actin, causing the failure of I-band assembly. The present data suggest that desmin is an important cytoskeletal component, not only in the determination of cell shape, but also in the orderly assembly and alignment of cardiac myofibrils and maintenance of myofibril structure integrity, and in the necessity of desmin filaments for titin assembly.

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