

The involvement of adherens junction components in myofibrillogenesis in cultured cardiac myocytes

EKATERINA J. GONCHAROVA¹, ZVI KAM² and BENJAMIN GEIGER^{2,*}

¹Department of Cell Cultures, Institute of Cytology of the Academy of Sciences of the USSR, Leningrad, USSR

²Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

* Author for correspondence

Summary

The distribution of adherens junction (AJ) components was investigated in cultured heart myocytes. These cells, derived from either newborn rats or chick embryos, develop elaborate arrays of myofibrils which become extensive and laterally aligned following several days in culture. The Z-disks in these cells, visualized by immunolabeling with antibodies to muscle-specific α -actinin, exhibit a characteristic periodicity of about 2 μ m and are in register with those of neighboring myofibrils throughout the sarcoplasm. Vinculin, in these cells, associates with intercellular AJ and cell-matrix adhesions. In addition, this protein is detected in periodic bands located along the lateral cell membranes corresponding to "costamers" previously described by Pardo, J.V., Siliciano, J.D. and Craig, S.W. (*Proc. Natn. Acad. Sci. USA*, 80, 1008). Similarly, N-cadherin, which is predominantly associated with intercellular junctions, is also detected in periodic striations located mainly on the dorsal and lateral cell surfaces. Using computer-aided three-dimensional microscopy con-

firmed that these vinculin- and N-cadherin-containing structures are located in extrajunctional sites, apparently associated with Z-disks of peripheral myofibrils. Based on these findings an alternative pathway is proposed for the assembly of vinculin and N-cadherin, which is not triggered by adhesive interactions with extracellular surfaces but rather by interactions at the membrane-cytoplasm interphase with the periphery of the pre-assembled myofibrils. Moreover, we present evidence that antibodies to N-cadherin, which are capable of blocking AJ formation in culture, have an inhibitory effect also on the development and alignment of myofibrils. We discuss the functional significance of the "costameric" organization of vinculin and N-cadherin and consider its involvement both in the lateral alignment of neighboring muscle cells and in the stabilization of developing myofibrils.

Key words: adherens junctions, cell adhesion, cadherin, myogenesis.

Introduction

The assembly of the contractile apparatus of cardiac muscle involves several developmental stages including the interaction between neighboring myoblasts via prominent *fascia* and *maculae adherentia*, progressive assembly of thin and thick filaments into cytoplasmic arrays and the subsequent appearance of aligned sarcomeric structures with characteristic and uniformly spaced Z-disks and M-lines (Fischman, 1967, 1970). Detailed examination of this process focused much attention on the interaction between myofibrils and the plasma membrane both during myofibrillogenesis and in the mature cells. It had been reported that nascent myofibrils assemble mostly in close proximity to the sarcolemma and then expand until they occupy most of the cell volume (Holtzer et al., 1959; Kelly, 1969; Dlugasz et al., 1984). The peripheral myofibrils appar-

ently interact with the membrane via an electron-dense plaque material while the more "medullary" myofibrils are associated with each other and with the sarcoplasmic reticulum, mostly at the level of the Z-disks (Cheisi et al., 1981). It had further been proposed that peripheral components of the Z-disk, including desmin-containing intermediate filaments and spectrin take part in these intermyofibrillar or myofibril-membrane associations (Granger and Lazarides, 1979; Lazarides, 1980; Goodman et al., 1981; Messina and Lemanski, 1989; Nelson and Lazarides, 1984). Cultured heart muscle cells have become, over the last decade, a most useful system for the study of myofibril assembly at both structural and molecular levels (Claycomb and Palazzo, 1980; Nag and Cheng, 1981). The examination of such cultures by immunofluorescence microscopic labeling revealed the spatial and temporal assembly of the different molecular components of myofibrils into the mature structure. This approach included the localization of a multitude of proteins such as actin, myosin, α -

actinin, tropomyosin, titin, tubulin and vinculin (Nag et al., 1983; Wang et al., 1988; Sanger et al., 1984; Terai et al., 1989; Guo et al., 1986; Lin et al., 1989; Schultheiss et al., 1990).

Especially intriguing was the distribution of vinculin in striated muscle. This $117 \times 10^3 M_r$ protein was previously shown to be ubiquitously associated with the cytoplasmic faces of all adherens type junctions (AJ) including intercellular junctions and cell-matrix adhesions (Geiger et al., 1981, 1985, 1987; BurrIDGE, 1986; BurrIDGE et al., 1990). In addition to its prominent association with intercellular junctions and with the termini of myofibrils, vinculin in muscle cells was also detected in periodically spaced "costameric structures" located at the vicinity of Z-disk attachment sites to the plasma membrane (Pardo et al., 1983a and b; Koteliatsky and Gneushev, 1983). Moreover, in view of the apparent involvement of vinculin in the membrane anchorage of actin filaments in cell junctions (Geiger, 1979; Geiger et al., 1981), it was assumed that this protein plays a similar role also in linking the periphery of Z-disks to the sarcolemma (Pardo et al., 1983b; Koteliatsky and Gneushev, 1983).

To define the relationships between myofibril assembly and membrane anchorage and to determine the involvement of AJ in these processes, we have examined the organization of two AJ components, vinculin and N-cadherin (A-CAM, Hatta et al., 1988; Geiger et al., 1990a), during various phases of cardiac myocyte development in culture. We have found that in addition to its prominent association with intercellular "fascia adherens-like" junctions (Tokuyasu et al., 1981), vinculin is detected along Z-disks of peripheral myofibrils which are closely associated with the cell membrane. This became evident from the examination of three-dimensional reconstructions of cultured muscle cells, double labeled for vinculin and actin. In addition, we observed a prominent association of N-cadherin with these "costameric" structures along peripheral Z-disks located mostly at the dorsal and lateral cell surfaces. Moreover, addition of N-cadherin antibodies against N-cadherin to cultured heart myocytes resulted in a marked inhibition of myofibrillogenesis. The basis for the localization of vinculin and N-cadherin in these extrajunctional areas and their possible involvement in muscle organization are discussed.

Materials and methods

Antibodies

(1) Monoclonal antibodies against A-CAM (N-cadherin) used here included ID 7.2.3 (Volk and Geiger, 1984) or GC-4 (Volk et al., 1990), both are now available from Sigma Immunochemicals (St Louis, USA).

(2) Anti-vinculin used here was a monoclonal antibody raised against human vinculin, kindly provided by Dr V. Koteliatsky from the Institute of Experimental Cardiology, Moscow, USSR.

(3) Rabbit polyclonal antibodies against desmin were purchased from Sigma Immunochemicals (St Louis, USA).

(4) Monoclonal antibodies specific for muscle α -actinin were as previously described (Fridlanskaya et al., 1989).

(5) Secondary antibodies used here were DTAF- and rhodamine-lissamine B-modified goat anti-mouse or -rabbit IgG (Jackson ImmunoResearch Labs PA, USA).

Cell culture

Heart muscle cell cultures were prepared from the ventricles of 1- to 3-day-old rats (Wistar strain) or of 7-day-old chicken embryos, using modification of previously described procedures (Nag and Cheng, 1981; Dlugasz et al., 1984). Ventricles were isolated under sterile conditions and washed in Hank's Ca^{2+} -free solution and again in phosphate-buffered saline (PBS). The tissue was minced and transferred into PBS containing 0.1% trypsin (Difco, USA) for 15-20 minutes at 37°C. After incubation with trypsin solution, the supernatants were discarded and the tissue further processed as follows. For the preparation of rat cardiac myocyte cultures, fresh PBS containing 0.1% trypsin and 0.1% collagenase 1A (Sigma, USA) was added to the tissue for 20 minutes. For chicken embryos, PBS containing 0.1% trypsin and 0.05% collagenase was added for 15 minutes. After exposure to the enzyme solutions, cells were collected by centrifugation (3-5 minutes at 1000 rpm in a table top centrifuge) and washed once in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine, 1% antibiotic-antimycotic solution (all purchased from Bio-Lab, Israel). Cells were gently resuspended in new medium and centrifuged for 2-3 minutes to remove large tissue fragments. Supernatants containing suspended cells were plated on 18 mm coverslips. Fresh enzyme solutions were added to the remaining undissociated tissue and the procedure was repeated 3-4 times. Cultures were incubated at 37°C in humidified atmosphere of 5% CO_2 and 95% air.

Immunofluorescence labeling

For immunofluorescence labeling with antibodies to α -actinin, vinculin, or N-cadherin (A-CAM), cells were permeabilized at room temperature by 2 minutes exposure to 0.5% Triton X-100 in 50 mM morpholinoethane sulfonate buffer, pH 6.0 and fixed for 30 minutes with 3% paraformaldehyde. For immunofluorescence labeling with anti-desmin antibodies, cells were fixed and permeabilized for 10 minutes with cold methanol ($-20^\circ C$) and then for 1 minutes with cold acetone ($-20^\circ C$). Incubations with antibodies were carried out on Parafilm sheets for 30 minutes at room temperature. Cells were rinsed in PBS after the incubations with the primary and secondary antibodies and mounted in Elvanol.

Immunofluorescence microscopy and three-dimensional reconstructions

Conventional immunofluorescence microscopy and photomicrography were carried out using Axiophot microscope (Zeiss, Oberkochen, FRG) equipped with filter sets for selective rhodamine/fluorescein fluorescence.

The computerized microscopic system used here was based on the design of Agard and Sedat (Agard et al., 1988, 1989). The system consisted of a Zeiss Axiomat microscope and microvax III workstation which controls image acquisition, light shutters and focus. Low-light-level images were recorded with a cooled, scientific-grade, charge coupled device camera (CCD, Photometrics, Arizona, USA, for details see Hiraoka et al., 1987). Images were read into a Mercury Array Processor (Mercury computer systems, Lowell MA, USA) which calculated on-the-fly pixel per pixel correction for illumination and CCD sensitivity, scaled and deconvoluted images essentially in real-time (Chen et al., 1990). Optical

sections of double-labeled slides were taken in the fluorescein and rhodamine channels with selective Zeiss filter sets.

The three-dimensional imaging and modeling package (PRISM, Chen et al., 1990, Kam et al., 1991) employs high-resolution (1280×1024, 60 Hz, non-interlaced) video board with 12 Mbytes of memory (Parallax Graphics, Inc. Santa Clara, CA USA), and implements a flexible multiple windowing architecture and interactive display of images series. Versatile color menus allow images of different fluorophore staining of the same sample to be superimposed. A digital movie of sequentially displayed tilt series produce a realistic perception of the three-dimensional distributions of the labeled molecules, and creates a powerful medium for studying complex colocalization of molecules. Pairs of images from such rocking projection series were selected for stereo presentations in this work. The three-dimensional aspect of the data is enhanced by three- to five-fold increase in the separation between sections. Pictures were photographed on Ektachrome 160 or 100/Day light diapositive film using Focus 4700 Imagecorder (Focus Graphics, CA. USA).

Results

Development of myofibrillar structure in cultured cardiac myocytes

In this study, we have examined heart cells from two sources, namely rat and chick, which develop in culture at different rates, and with distinct morphology. Since development of cultured cardiac myocytes was previously described, we will refer here only to major stages in myocyte development. Isolation of cardiac myocytes of 7 day chicken embryos and their transfer to culture resulted, initially, in a dramatic deterioration of sarcomeric structures. Up to the third day after plating only some myofibrils were found in the cytoplasm while from the third day and on, a progressive increase was noted in the number of striated myofibrils, their size and their alignment (Dlugasz et al., 1984; Sanger et al., 1986; see also Fig. 1). At first stress fiber-like structures were frequently noted by phalloidin staining which were decorated by bead-shaped α -actinin-containing structures. These cells were clearly identified as developing muscle cells in as much as they were labeled with the muscle-specific anti- α -actinin antibody and often detected in the same cells as organized myofibrils. The early myofibrils, identified by the typical periodicity of $\sim 2 \mu\text{m}$, were of variable size and usually poorly aligned. Following longer incubation in culture, the stress fiber-like filament arrays became less prominent concomitantly with a marked increase in myofibril alignment and a moderate increase in their number and size, with α -actinin located with their Z-disk.

In rat cardiomyocyte cultures, discrete sarcomeric structures were noted already one day following plating, though the cells were usually small and the myofibrils short and poorly aligned (see Fig. 2A). Upon longer incubation in culture, the cells underwent considerable spreading and differentiation showing conspicuous increase in the content and alignment of myofibrils (Fig. 2C). At the termini, myofibrils were often associated with stress-fiber-like actin-containing structures displaying beaded α -actinin distribution.

These filament bundles terminated at the ventral cell surface, in an apparent association with focal contacts (see also Guo et al., 1986 and Fig. 2).

Organization of vinculin in cultured cardiac myocytes

At early stages of myofibrillogenesis in the chick (3 days in culture, Fig. 1A,B), close association between actin-containing fibers and vinculin was noted. Unlike non-muscle mesenchymal cells, in which vinculin was associated predominantly with focal contacts located at the termini of stress fibers (the arrowheads in Fig. 1A,B point to focal contact-associated vinculin in a fibroblast present in the culture) prominent beaded vinculin staining was detected along the developing myofibrils with an apparent average periodicity of about $1 \mu\text{m}$ (Fig. 1A,B, arrows). Upon longer incubation (6 days), vinculin staining was detected both at the termini of myofibrils and along their length where it was associated with many (but apparently not all) Z-disks (Fig. 1C,D). This was verified by careful alignment of the actin- and vinculin-labeling (see for example matched arrow in Fig. 1C,D). It is noteworthy that vinculin antibodies did not react with other structures periodically located along the myofibrils.

The apparent association of vinculin with Z-disk area was even more conspicuous in rat cardiomyocyte cultures. The presence of vinculin in both substratum adhesions and Z-disks was already clear following 3-day in culture (Fig. 2A,B) and became more extensive after incubation for additional 3 days (Fig. 2C,D). Often, when groups of cells were examined, additional labeling was noticed along the intercellular junctions formed between adjacent cardiac cells (two muscle cells or one muscle cell and one fibroblast) adopting a typical "intercalated disc-like" organization (Fig. 2F). Additional analysis indicated that another component of the Z-disk periphery, namely desmin was already associated with Z-disks following 3-days in culture. Comparison of desmin staining to that of α -actinin and vinculin suggested that α -actinin is associated with Z-disks earlier than the other two proteins and that desmin staining along Z-disks appears earlier and is more extensive than that of vinculin (Fig. 2E,F). It was however clear that, whenever present, the labeling of desmin and vinculin was overlapping.

To explore the possibility that vinculin is selectively associated only with Z-disks of "peripheral" myofibrils (i.e. those located immediately subjacent to the plasma membrane), we have determined the relative distributions of vinculin and actin in the same developing muscle cells by examination of optical sections produced by computer-aided microscopy. We have selected to examine cultured rat cardiomyocytes after 3 days in culture. At that stage, prominent association of vinculin with the Z-disk area was already detected, yet the cells were moderately spread and thus allowed for distinction between myofibrils located at different focal levels. As evident from the optical sections of the double-labeled cell (Fig. 3), most of the ventral actin-containing myofibrils were associated with periodic vinculin striations (Fig. 3A,B) while in sections located

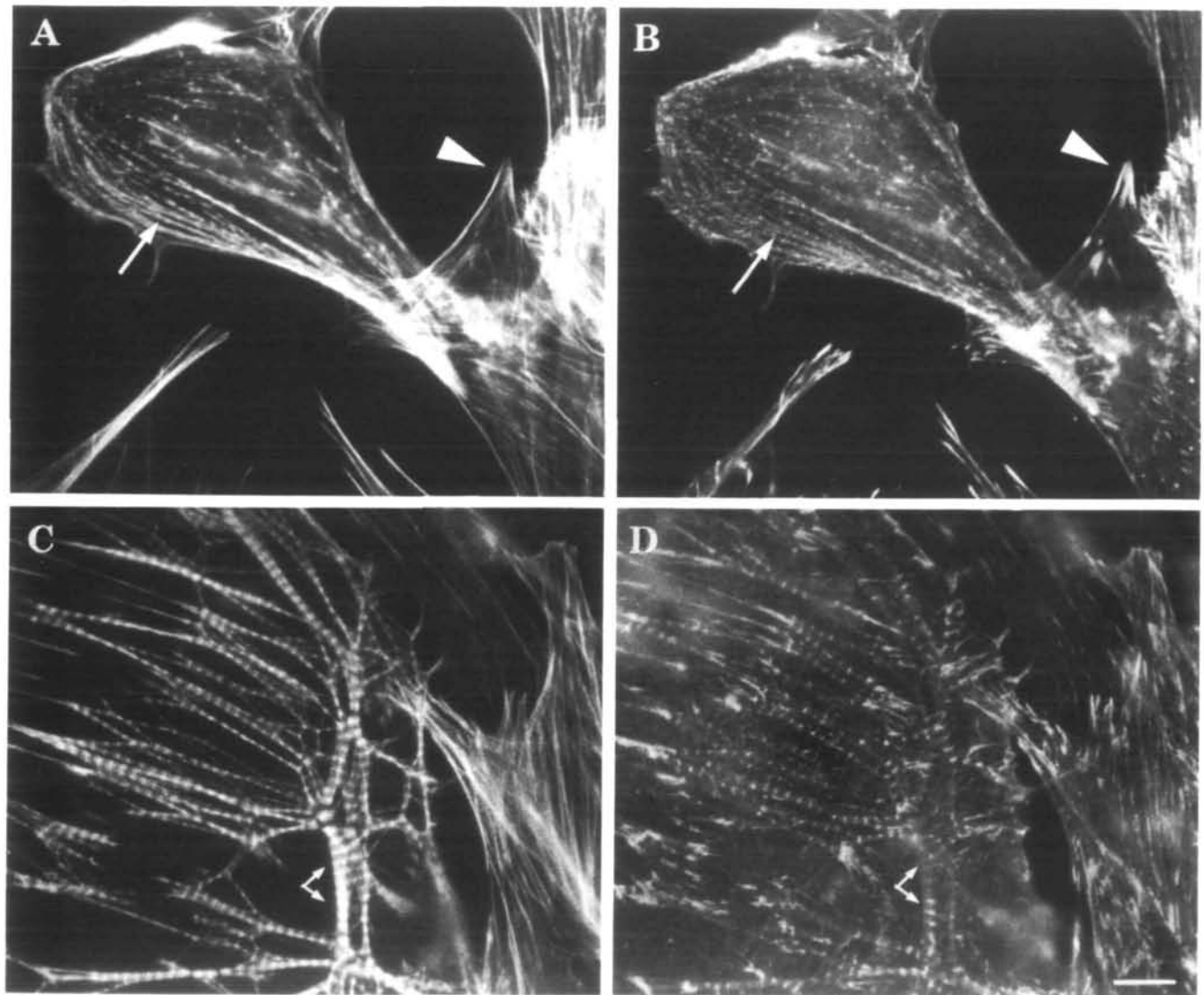


Fig. 1. Immunofluorescent localization of actin (A,C) and vinculin (B,D) in chicken cardiomyocytes cultured for 3 days (A,B) or 6 days (C, D). Note the presence of vinculin both in focal contacts and in an apparent association with some of the Z-disks. The matching paired-arrows point to regions along the same myofibril that exhibit highly variable levels of vinculin staining. The bar indicates 10 μ m.

at the more dorsal levels, most myofibrils (except for the most peripheral ones) were cleared of vinculin (Fig. 3C,D).

Distribution of N-cadherin (A-CAM) in developing chicken cardiomyocytes

Immunolabeling of cultured chick myocytes with anti-N-cadherin antibodies yielded intense staining of intercellular contacts, in accordance with our previous results (Volk and Geiger, 1984). This junctional staining pattern was detected already one day after plating (Fig. 4A) and persisted in the same culture for 7-10 days. However, it was noted that, in addition to the labeling at cell-cell contacts, periodically spaced spots and bands were also present, located along the ventral, dorsal or lateral surfaces of the cells. This was first noted at 3 days

after plating (Fig. 4B) and became more prominent by 6 days (Fig. 4C).

To obtain a more accurate view of the spatial relationship between N-cadherin and actin, we have examined cells displaying different stages of myofibrillar organization using the computer-aided three-dimensional microscopy. Fig. 5 shows cultured heart muscle cells which were fixed before extensive spreading had occurred. In these cells, some actin bundles and primordial myofibrils are detected throughout the cytoplasm. The N-cadherin labeling appears in two major forms in these cells: long, nearly continuous-line running along the cell-cell junction and spots, scattered over the cell periphery. Examination of the overlaps between cadherin and actin labeling (represented by overlapping blue and yellow areas which appear white in these micrographs) indicated that most of these

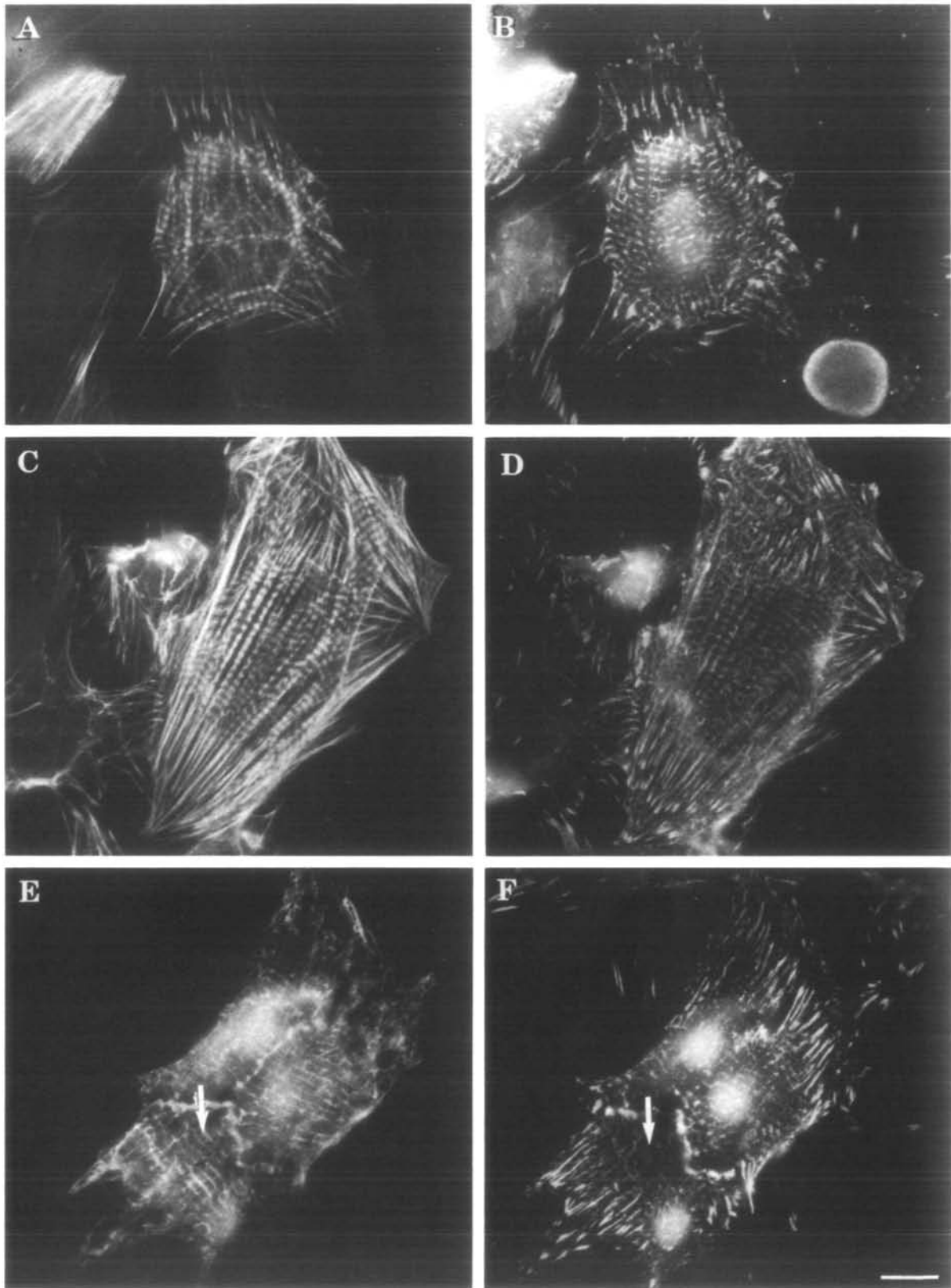


Fig. 2. Immunofluorescent localization of actin (A,C), vinculin (B,D,F) and desmin (E) in rat cardiomyocytes cultured for 3 (A,B,E,F) or 6 days (C,D). Notice the prominent association of vinculin labeling with focal contacts, intercellular AJ and Z-disks at both early and advanced stages of development and the more extensive labeling for desmin along the latter two areas (the matched arrows in E and F point to desmin-containing Z-disks which appear to be devoid of vinculin). The bar indicates 10 μ m.

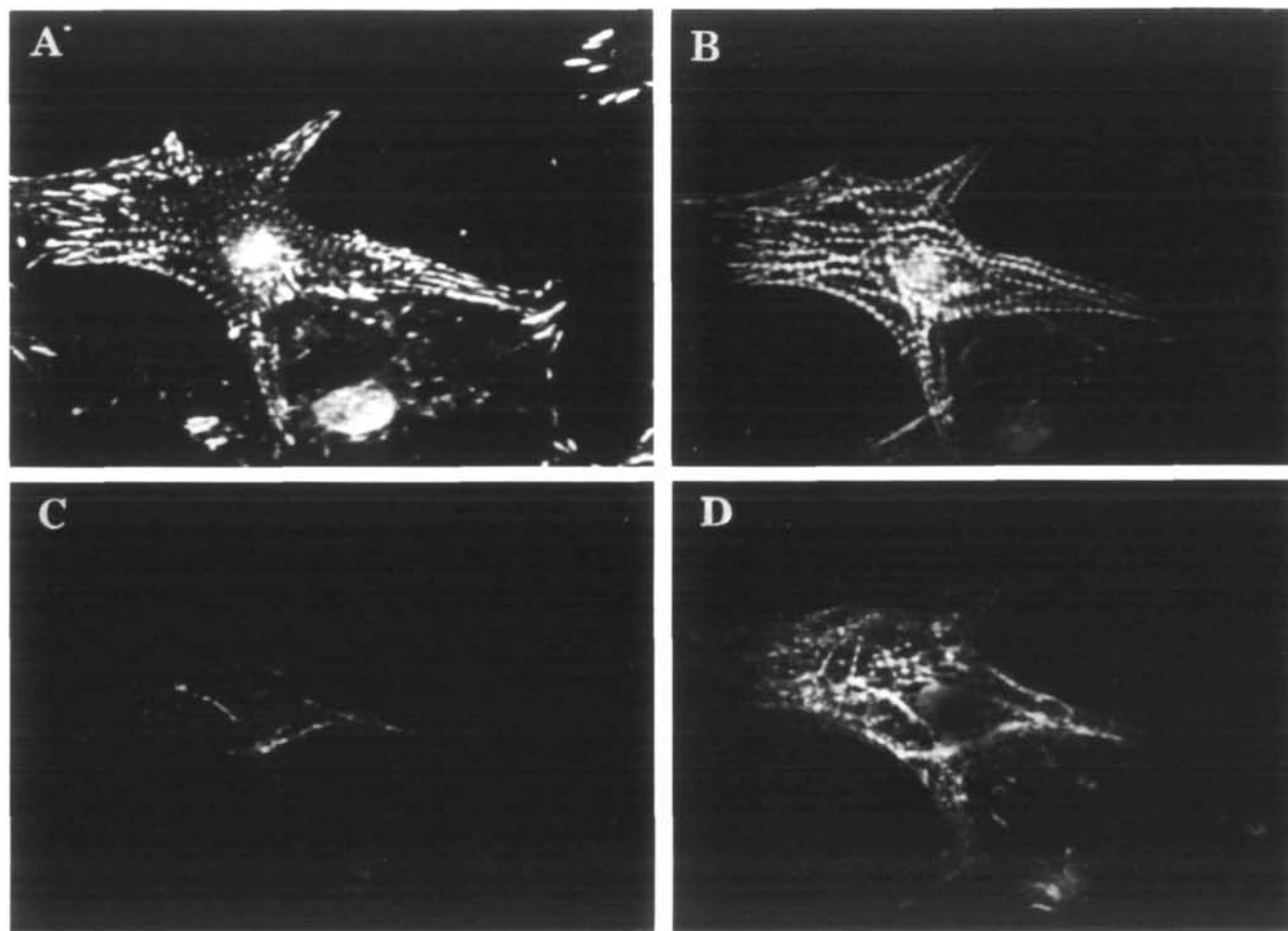


Fig. 3. Optical sections of 3-day cultured rat cardiomyocytes, double-labeled for vinculin (A,C) and actin (B,D). The focal planes selected are ventral (A,B), and close to the dorsal surface (C,D). Notice that the majority of actin-containing ventral myofibrils are associated with vinculin at their termini and along their sarcomere, while closer to the dorsal surface only cortical myofibrils are positive for vinculin. Bar indicates 10 μm .

cadherin-containing spots were, in fact, associated with actin. On the other hand, the myofibrils which were not located at the cell periphery were apparently devoid of N-cadherin labeling.

As pointed out above, at more advanced stages of development, N-cadherin-labeled striations become apparent. The relationship of these structures to the myofibrillar network was determined by computerized optical sectioning analysis as shown in Fig. 6, as well as by careful examination of flatly spread cells in which the images were essentially two dimensional (Fig. 7). The three-dimensional reconstitution of the N-cadherin/actin double-labeled cells is shown in the bottom panel (Fig. 6G,H). Stereoscopic examination showed that cadherin labeling (which appears yellow or white - when superimposed on the "blue" actin) is mostly restricted to the periphery of the cells and is present in the form of long streaks located at cell-cell contact regions, scattered spots and uniformly spaced striations with $\sim 2 \mu\text{m}$ periodicity. The latter were often located on the dorsal cell surface with no apparent relationship to intercellular junctions. Since the reconstituted images of whole cells seem too complex for detailed

spatial analyses, we have selected a narrower range of optical sections located near either the ventral or the dorsal focal planes of the cells. Examination of the superimposed images (Fig. 6B,E) displaying N-cadherin (yellow) (Fig. 6A,D) and actin (blue) (Fig. 6C,F) indicates that the N-cadherin labeling close to the ventral area was mostly restricted to cell-cell contacts while in dorsal areas these striations were apparently associated with the control region of the I-band along the sarcomeric actin. This notion was further supported by two lines of evidence. Linear computer scanning along the myofibrillar axis confirmed that the periodicities of actin and N-cadherin were 2.1 ± 0.2 and $1.93 \pm 0.2 \mu\text{m}$, respectively. There was however large difference in the width of the labeled bands. The average of full width of actin bands at half peak intensity was $1.15 \pm 0.2 \mu\text{m}$ while the average width of cadherin bands was only $0.47 \pm 0.2 \mu\text{m}$. High resolution examination of cultured chick cells labeled for N-cadherin and either α -actinin or actin confirmed that the cadherin labeling was indeed associated with the Z-disc area (Fig. 7).

Periodic organization of N-cadherin at or near Z-disk-membrane contacts is not exclusively present in

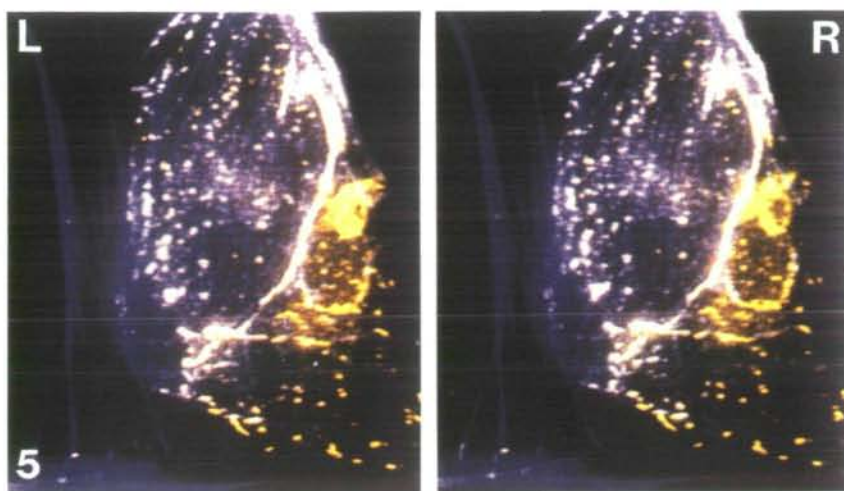
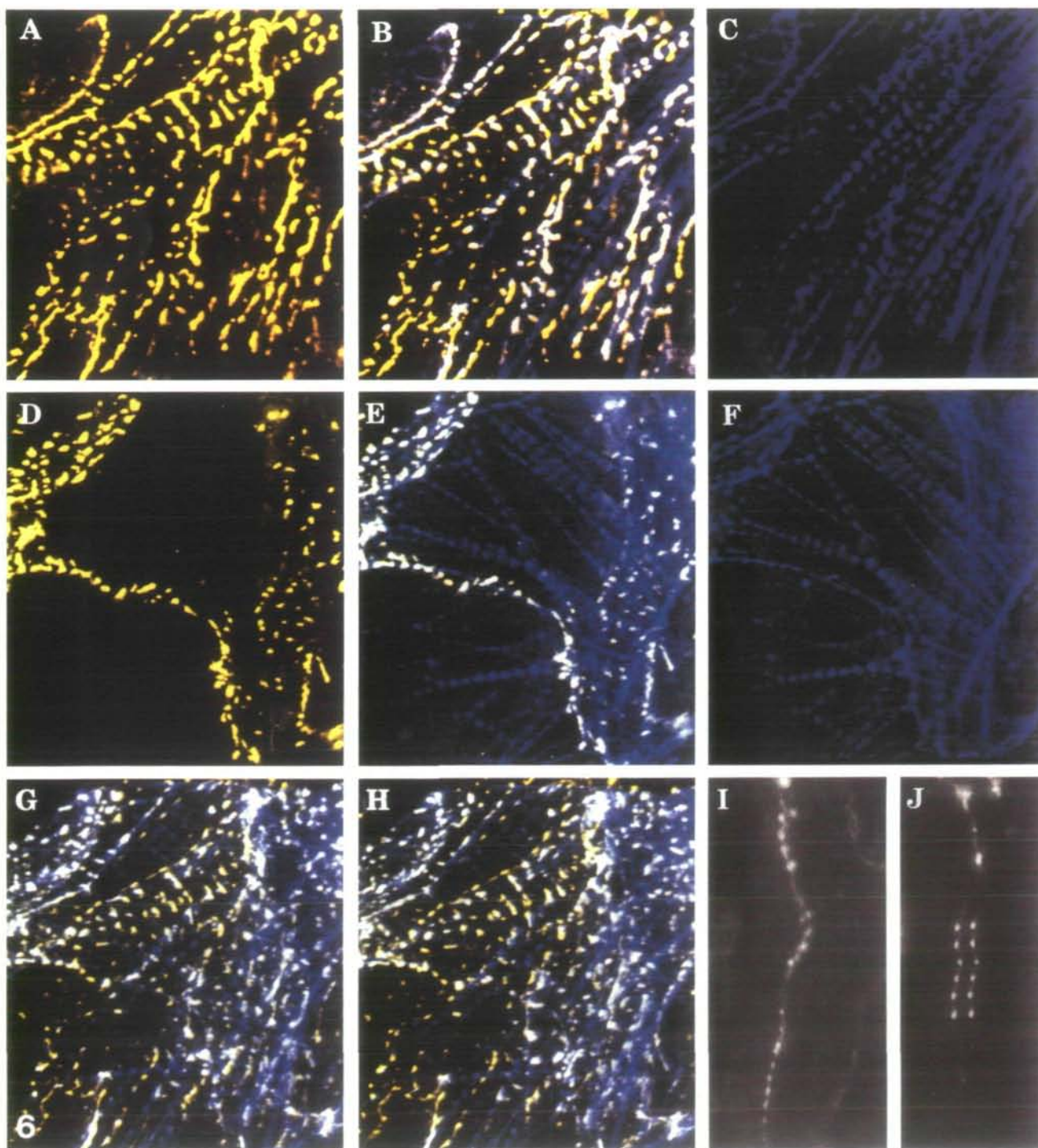


Fig. 5. Computer-generated stereoscopic view of 2-day chick cardiomyocytes, double-labelled for actin (blue) and N-cadherin (yellow). Notice that N-cadherin is predominantly associated with actin in the intercellular junction with only sporadic patches along the cell surface.

Fig. 6. Computer-generated optical sections (A-F) and stereoscopic view (G,H) of differentiated chick cardiomyocytes, double-labelled for actin (blue) and N-cadherin (yellow). The free dorsal cell surface is depicted in A-C, showing overlapping strations (white in panel B) containing both proteins along peripheral myofibrils. At the ventral focal plane (D-F), close to the substratum, N-cadherin is apparently absent from the myofibrils and detected mostly at cell borders. The three-dimensional relationships between the two proteins are displayed in the stereopair (G,H). Distribution of N-cadherin in chick cardiac muscle indicates periodic association of the label with the periphery of Z-disks (I). Occasionally, when two adjacent cells retracted during processing, it became evident that the N-cadherin patches in neighboring cells are in register (J).



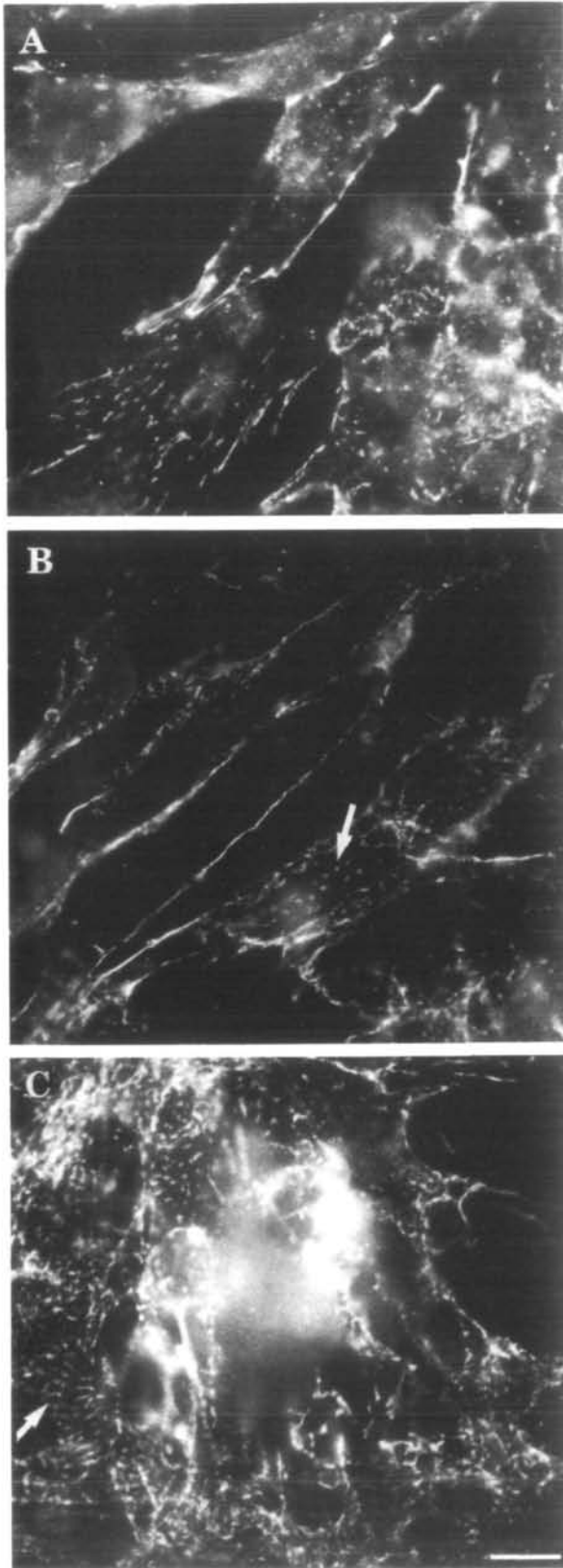


Fig. 4. Immunofluorescent localization of N-cadherin in chicken cardiomyocytes after 1 (A), 3 (B) and 6 days (C) in culture. Initially, labeling is restricted to cell-cell contacts whereas at more advanced stages punctate staining, corresponding to sarcomeric periodicity, is evident (see areas indicated by arrows in B and C). Bar represents 10 μ m.

displayed a typical sarcomeric periodicity and was apparently in register with peripheral Z-disks (Volk and Geiger, 1984, see Fig. 5A). An example of frozen sections of adult chicken heart following immunofluorescent labeling with anti-A-CAM/N-cadherin antibodies is shown in Fig. 6I,J, indicating that this protein is indeed present along Z-disks, supporting the observations made with cultured myocytes.

Effect of anti-N-cadherin on myofibrillogenesis in culture

In view of the apparent association of N-cadherin with the periphery of Z-disks, we have explored the possibility that this protein is involved in myofibrillogenesis. For that purpose chicken cardiac myocytes were plated on glass coverslips in the presence of purified IgG fraction of monoclonal anti-A-CAM antibody (clone GC4), which was previously found to be an effective inhibitor of adherens junction formation in cultured chick lens epithelial cells (Volk and Geiger, unpublished results). As a control, we have used class-matched monoclonal antibodies directed against IgE. In both cases, the IgG fraction was isolated by DEAE-cellulose chromatography and used at final concentrations of 0.2-0.4 mg/ml. The cells were incubated in culture for 2 days, then fixed and immunolabeled for α -actinin to examine the extent of myofibrillogenesis.

As shown in Fig. 8A, incubation with the control antibodies, even at a relatively high concentration, did not impair the development of elaborate myofibrillar arrays. Anti-A-CAM, on the other hand, strongly inhibited the development of α -actinin containing myofibrils. This was manifested by poor alignment of the filament bundles and the presence of "beaded" rather than sarcomeric structures. This effect appeared to depend on the concentration of inhibitory antibodies (compare Fig. 8B,C) and was readily reversible: replacement of anti-A-CAM with regular medium resulted in the re-acquisition of typical myofibrillar organization within 24 hours (Fig. 8D).

Discussion

In this study, we have examined the subcellular distribution of the AJ components vinculin and N-cadherin in cultured cardiomyocytes at different stages of myofibrillogenesis. The observations described here indicated that these molecules are present in two distinct cellular sites: the first are intercellular AJ and the other are membrane regions associated with cortical myofibrils.

cultured heart cells. In fact, in the first report on A-CAM distribution, we have noted that, in heart tissue, this protein was associated not only with intercalated disks but also with lateral membranes, where it often

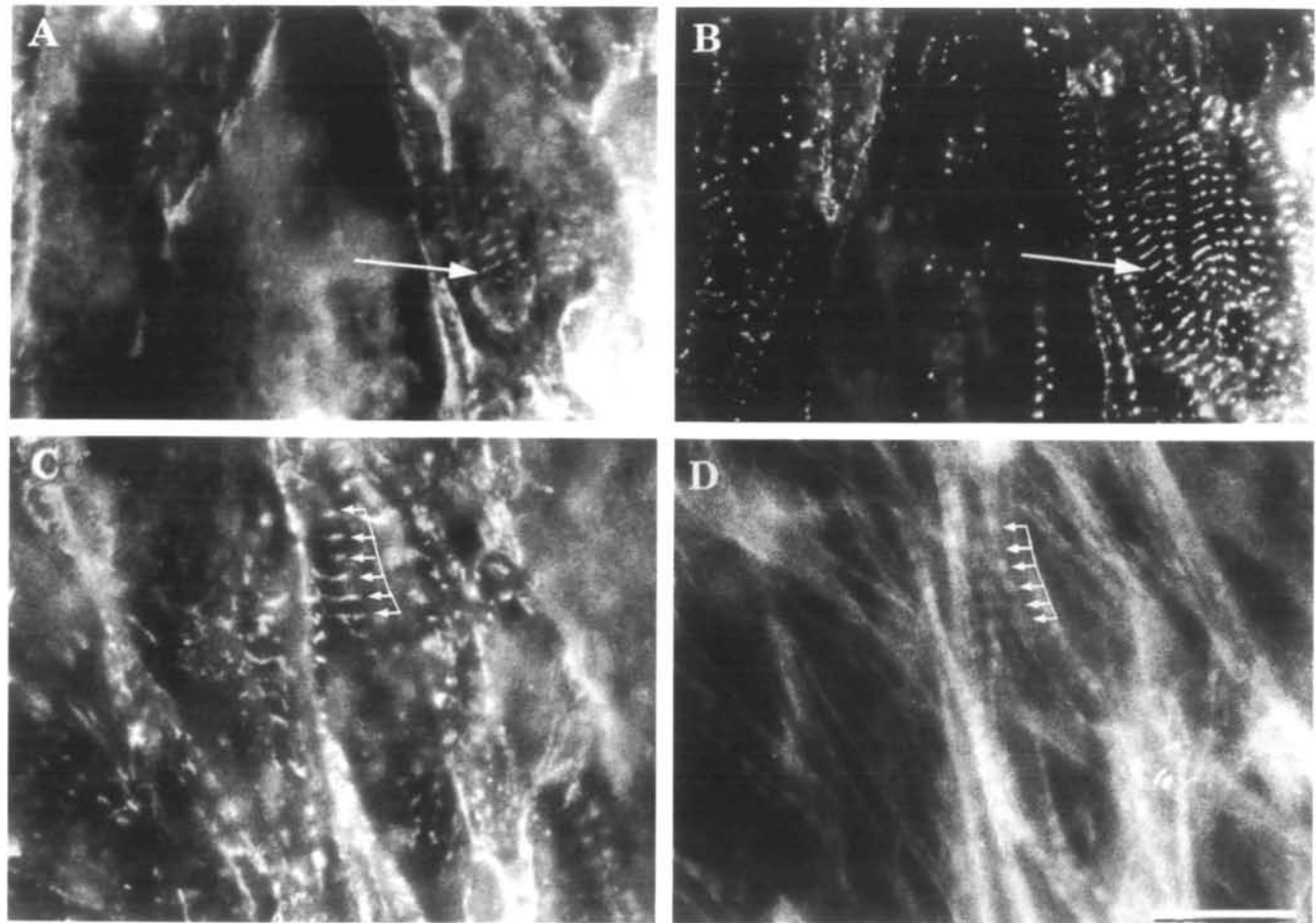


Fig. 7. Double immunofluorescent localization of N-cadherin (A,C) and either α -actinin (B) or actin (D). The arrows point to identical location. Notice that N-cadherin is associated with sharply delineated bands, superimposed on α -actinin striations, and the central region of the I-bands. Bar represents 10 μ m.

The presence of AJ in heart muscle has been amply documented in recent years. It had been shown that vinculin, α -actinin and A-CAM (N-cadherin) are enriched along the *fascia adherens* in cardiomyocytes both in culture and in situ (Tokuyasu et al., 1981; Koteliensky and Gneushev, 1983; Volk and Geiger, 1984; Geiger et al., 1990a). The assembly of these and other components into a mature AJ has not been well characterized at the molecular level. Yet it had been widely accepted that this process is initiated by immobilization of the relevant receptors (cadherins in cell-cell AJ) followed by assembly of the submembrane plaque and cytoskeletal proteins (Geiger et al., 1985, 1987, 1990b). This was mainly based on the observation that AJ-specific molecules are rarely detected in extrajunctional sites and that cell-cell and cell-matrix adhesions appear to act as nucleation sites for the organization of the microfilament system. The only observation that was apparently inconsistent with this view is the finding of organized vinculin in extrajunctional "rib shaped" sites in muscle cells known as "costameres" (Pardo et al., 1983a,b, see also Fig 5b in Schultheiss et al., 1990). The mechanism of costamer formation and their physiological roles are, however,

still obscure. In the present study, we have extended the characterization of costamers and showed that not only cytoplasmic components of AJ such as vinculin but also the respective membrane receptor, namely N-cadherin, becomes associated with myofibrillar structures in differentiating cardiomyocytes. This organized N-cadherin, displayed as regularly spaced dots or bands, had a rather uniform periodicity of about 2 μ m, which was essentially identical to that found in muscle sarcomeres. Furthermore, it was noted that N-cadherin was colocalized with only a limited fraction of the myofibrils. It was therefore important to determine what are the special features of that subpopulation of myofibrils which colocalized with N-cadherin and to which substructures along the sarcomere was the cadherin apparently bound.

To address the former issue, we have used the computerized three-dimensional microscopic examination of cells, double labeled for both actin and N-cadherin. Careful plane-by-plane examination of these specimens, along with the phase-contrast or Nomarski images, revealed the relative topologies of the two molecule. Their spatial interrelationships indicated that only myofibrils located at the cell periphery exhibit such

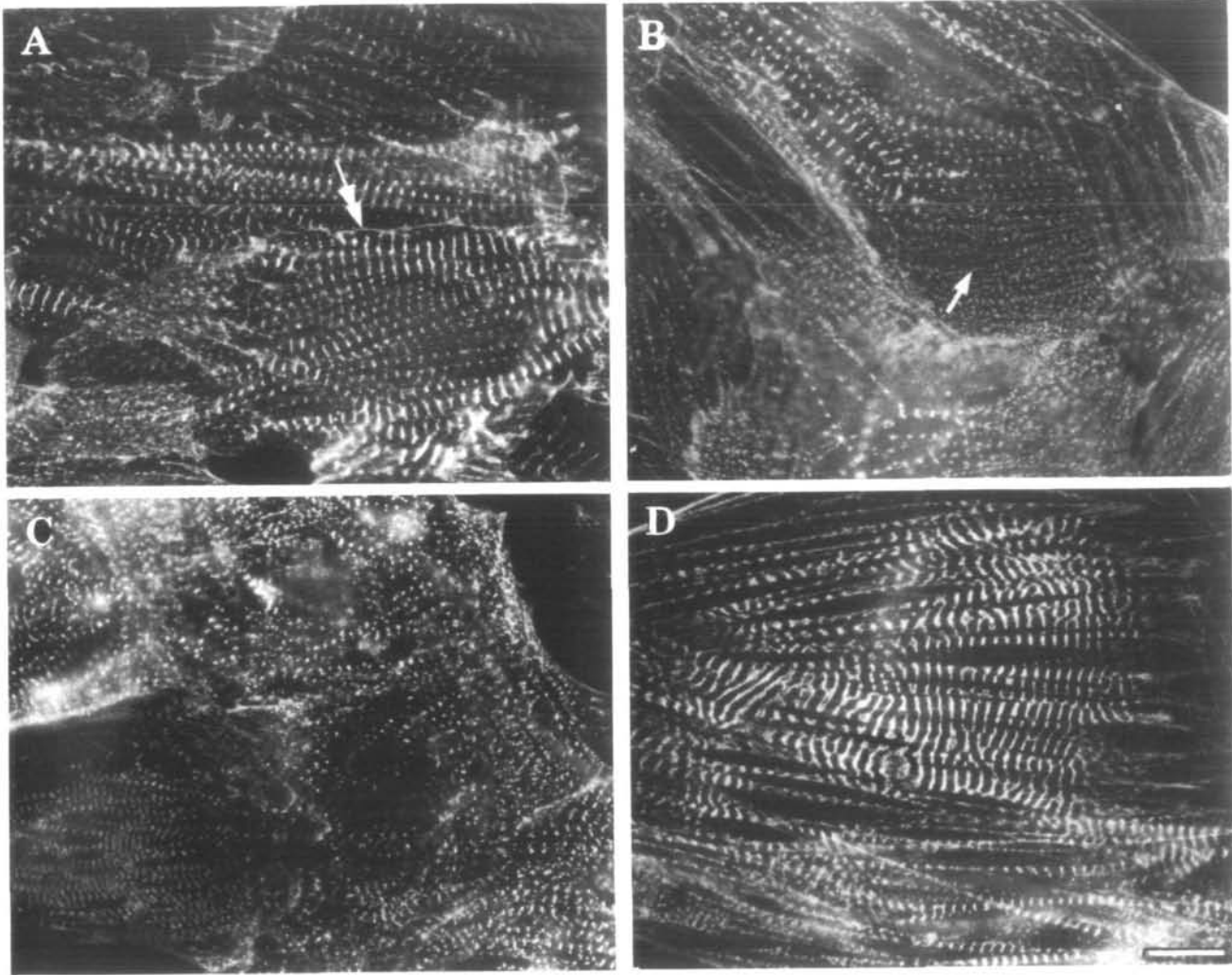


Fig. 8. Effect of anti-N-cadherin antibodies on myofibril development. Chicken cardiomyocytes were plated in culture in the presence of 0.4 mg/ml of an irrelevant mouse IgG (A), or anti-N-cadherin (0.2 mg/ml- B or 0.4 mg/ml- C). Following two days in culture, cells were fixed and labeled for α -actinin. Notice that in the control cells α -actinin was present both along the Z-disks and intercellular junctions (double-headed arrow in A while myofibril assembly was markedly inhibited by the treatment with anti-N-cadherin as apparent from the reduction in the number and size of Z-disks and the presence of α -actinin-containing "beaded" filaments (arrow in B). This inhibition was reversible since fully developed Z-disks appeared within one day after removal of the inhibitory antibodies (D). The bar indicates 10 μ m.

association with cadherin and that the transverse N-cadherin bands were largely in register with Z-disks. Electron microscopic analyses are now in progress to assess these relationships at a molecular resolution. It is noteworthy that while this co-distribution was studied here primarily in differentiating cultured cells examination of cadherin distribution in intact chicken cardiac muscle using either A-CAM-specific or pan-cadherin antibodies indicated that prominent labeling was located at the periphery of Z-disks (Fig. 8, see also Fig 5a in Volk and Geiger, 1984, and Figs 4 and 5 in Geiger et al., 1990).

The molecular mechanism leading to this extrajunctional organization of N-cadherin is not clear. However, it is conceivable that the molecular interactions that link together the various constituents in intercellular AJ are also responsible for these extrajunctional assemblies.

Biochemical studies characterizing these interactions are mostly available for the cell-matrix AJ where it was shown that integrin can bind talin (Horwitz et al., 1986), or α -actinin talin may bind to vinculin (Burridge and Mangeat, 1984) and vinculin to α -actinin (Otto, 1983; Wilkins et al., 1983; Belkin and Kotliansky, 1987; Wachsstock et al., 1987) and paxillin (Turner et al., 1990) which is believed to be regulatory molecule. α -actinin has been known, for a long time, to bind and cross-link actin (Maruyama and Ebashi, 1965) and was recently shown to directly bind to integrin (Otey et al., 1990) and to another AJ constituent, zyxin (Crawford and Beckerle, 1991). It is likely that a similar chain of interactions exists also in cell-cell AJ (see, for Discussion: Geiger and Ginsberg, 1991). These binding data left, however, unresolved the intriguing question: how is the directionality of AJ assembly determined? In

the common molecular models for AJ formation, it was proposed that surface interactions, mediated by cadherins (in cell-cell AJ), or integrins (in cell-matrix AJ), lead to the sequential local accretion of the cytoplasmic components of the junctional plaque (such as vinculin) and subsequently to the assembly of actin and associated proteins at the junctional site. The present results, on the other hand, raise the possibility that the organization of AJ constituents may be a bidirectional process, namely triggered not only by proper nucleation sites provided by extracellular multivalent ligands (a contact with an external surface), but also by pre-existing cytoskeletal structures. The presence of such "reverse assembly" is suggested in the present studies by the progressive organization of N-cadherin in non-junctional regions where no external organizer is detected. It is thus conceivable that the localization of both vinculin and N-cadherin in such sites, in close proximity to Z-disks of peripheral myofibrils is driven by a chain of interactions directed from pre-assembled intracellular structures towards the periphery.

Does the regular organization of N-cadherin along the lateral borders of cardiac myocyte have a functional significance? One tempting speculation is that the presence of "contact receptors", which are believed to be capable of mediating homophilic cell-cell adhesions, may contribute to the proper register of laterally neighboring muscle cells. Such paracellular alignment, effectively bridging between the peripheries of Z-disk in neighboring cells, may play an important role in efficient and coordinated contractility. Furthermore, the inhibition experiments shown in Fig. 8 support the notion that surface-associated N-cadherin may indeed play an important role not only in the establishment of intercellular contacts but also in myofibrillar organization.

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