Defects in Cell Adhesion and the Visceral Endoderm following Ablation of Nonmuscle Myosin Heavy Chain II-A in Mice*§

Mary Anne Conti%%§, Sharona Even-Ram§, Chengyu Liu**, Kenneth M. Yamada‡, and Robert S. Adelstein*

From the $Laboratory of Molecular Cardiology, NHLBI, the ¶Craniofacial Developmental Biology and Regeneration Branch, NIDCR, and the **Transgenic Core Facility, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

Previous work has shown that ablation or mutation of nonmuscle myosin heavy chain II-B (NMHC II-B) in mice results in defects in the heart and brain with death occurring between embryonic day 14.5 ($E14.5$) and birth (Tullio, A. N., Accili, D., Ferrans, V. J., Yu, Z. X., Takeda, K., Grinberg, A., Westphal, H., Preston, Y. A., and Adelstein, R. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12407-12412). Here we show that mice ablated for NMHC II-A fail to develop a normal patterned embryo with a polarized visceral endoderm (TE) and die by E7.5. Moreover, A/-A- embryoid bodies grown in suspension culture transiently shed cells. These defects in cell adhesion and tissue organization are explained by loss of E-cadherin and $\beta$-catenin localization to cell adhesion sites in both cell culture and in the intact embryos. The defects can be reproduced by introducing siRNA directed against NMHC II-A into wild-type embryonic stem cells. Our results suggest an essential role for a single, specific nonmuscle myosin isoform in maintaining cell-cell adhesions in the early mammalian embryo.

Vertebrate nonmuscle myosins II have been shown to play important roles in a variety of cellular processes such as cell motility, morphology, and cytokinesis (1, 2), but the specific roles of the nonmuscle myosin II isoforms during embryonic development are still under study. Three different isoforms of mammalian nonmuscle myosin heavy chains (NMHCs) have been described, each of which is widely distributed throughout the adult organism (3). While the human genes are referred to as MYH9, MYH10, and MYH14, the protein products are commonly referred to as nonmuscle myosin II-A, II-B, and II-C, deriving their names from the relevant NMHC. Ablation and mutation of NMHC II-B results in major structural abnormalities in the heart including a ventricular septal defect, mislocation of the aorta (16), and a defect in cytokinesis involving the cardiac myocytes (4). Brain defects include the abnormal migration of specific groups of neurons and a severe hydrocephalus (5).

Until now, the effects of ablating NMHC II-A have not been reported, although humans with a single amino acid mutation have been shown to manifest a number of abnormalities affecting the kidneys, platelets, lens, and inner ear (6). In this paper, we address the role of NMHC II-A during early mouse development and show that it plays an important role in cell-cell adhesion and the formation of a polarized visceral endoderm.

EXPERIMENTAL PROCEDURES

The following experimental procedures are described in the supplemental material: generation of A-A- mice, RNA analysis and immunoblotting, embryo dissection and immunohistochemistry, TUNEL and BrdUrd assays, real-time PCR, and the antibodies used in immunoblot analysis, immunohistochemistry, and immunofluorescence microscopy.

RT-PCR—cDNA was synthesized from RNA made from wild-type, A/-A- and A/-A- embryoid bodies using the Superscript I kit (Invitrogen) with 5 $\mu$g of RNA and random hexamer primers. Dilutions of cDNA from 1:20 to 1:80 were then amplified with specific primers (7).

Preparation and Plating of ES Colonies—ES cells (5 x 10^5) were plated on sterile glass coverslips in 15% ES cell-qualified fetal bovine serum (FBS; Invitrogen) DMEM supplemented with LIF (10 $\mu$g/ml, Chemicon, Temecula, CA) and cultured for 2 days in a 10% CO_2 incubator to form colonies. Colonies were fixed and permeabilized with 4% paraformaldehyde and 0.5% Triton X-100 in 5% sucrose in PBS, rinsed with PBS, and treated with M.O.M. blocking solution (Vector Laboratories, Burlingame, CA).

Preparation of Embryoid Bodies and Time-lapse Microscopy—Embryoid bodies were formed from either A-A- A/A- or wild-type ES cells and cultured according to Robertson (8). Cells (5 x 10^5 cells) were cultured in DMEM + 10% ES cell-qualified FBS (Invitrogen) without LIF for 48 h on bacteriological nonadhesive plates. The embryoid bodies were collected, washed, and transferred to a regular tissue culture dish for 2 h prior to filming, and phase contrast (Zeiss Axiovert 25, Jena, Germany) time-lapse images were captured every 10 min for 20-24 h using MetaMorph 6.12 software (Universal Imaging Corp., Downingtown, PA).

siRNA Electroporation into ES Cells—A pool of siRNAs (25 pmol) specific for mouse myosin II-A (SMARTpool, Dharmacon Research, Inc., Lafayette, CO) was electroporated into ES cells using the Avanti Nucleofector instrument and program A23 (Avanti Biosystems, Gaithersburg, MD). Cells were plated in 12-well plates for immunoblot analysis and in 6-well plates on 20-mm coverslips for immunofluorescence analysis. The vector pCX-EGFP (a gift of Andras Nagy, Toronto, Canada) was used as a control for electroporation efficiency (90%). Cells were cultured for 24-72 h prior to analysis.

RESULTS AND DISCUSSION

The NMHC II-A gene was disrupted by homologous recombination using the strategy shown in supplemental Fig. S1 to generate heterozygous mice (see Fig. 1A, lanes 5-7). To generate A-A- ES cells that would be useful for cell and developmental studies, one of the heterozygous ES cell clones was re-electroporated with the original construct and selected at 2.5 mg/ml G418. Southern blot analysis of A-A- ES cell clones is shown in Fig. 1A, lanes 3 and 4. Analysis of RNA from A-A-
and A\(^{+/−}\) ES cells identifies a band at the expected size (7.2 kb) for the NMHC messenger RNA in the A\(^{+/−}\) cells. No NMHC II-A mRNA is detected in the A\(^{-/-}\) ES cells (Fig. 1B).

Incubation of immunoblots of ES cell extracts with antibodies to NMHC II-A detected the heavy chain (200 kDa) in wild-type and A\(^{+/−}\) ES cells, but no protein was detected in the A\(^{-/-}\) ES cells. Cells of all three genotypes express NMHC II-B at approximately the same level, but none of the ES cell genotypes expresses NMHC II-C (Fig. 1C).

Heterozygous mice were indistinguishable from wild-type littermates. Because no A\(^{+/−}\) mice were born, embryos were dissected at various stages of development to determine at what stage the null mutation became lethal. No viable A\(^{+/−}\) embryos were found later than E7.5. In an effort to gain insight into the cause of the early lethality, we analyzed sections of mouse embryos between E5.5 and E7.5 for cell proliferation using BrdUrd staining and for apoptosis using TUNEL assays. There was no significant difference between normal and null embryos in either assay (see supplemental Fig. S2).

Fig. 2A shows the results of immunostaining normal and mutant embryos at E6.5 with antibodies to NMHC II-A and II-B, and Fig. 2B shows staining by antibodies to the transcription factor GATA-4. In normal embryos (Fig. 2A, panels d–f), antibody staining for NMHC II-A is found in all cell layers (Fig. 2A, panel e). A\(^{-/-}\) embryos were identified by a lack of staining with the NHMC II-A antibody and marked cellular disorganization (Fig. 2A, panels a and b). Staining with an antibody to NMHC II-C confirmed its absence at E6.5 (data not shown). It is of particular note that, although both the null and normal embryos stain with an antibody for NMHC II-B (Fig. 2A, panels c and f), this staining is absent or very low in the outer cell layer of the normal embryo, the visceral endoderm (Fig. 2A, panel f, bracket). Moreover, the shape of the cells forming the visceral endoderm differs markedly between normal and A\(^{-/-}\) mice, with development of a polarized columnar morphology in the former by E6.5 (Fig. 2A, panels d and e, bracket, enlarged in inset), while a cuboidal shape is retained in the case of A\(^{-/-}\) mice (Fig. 2A, panel a, and Fig. 2B, panel a, arrows).

To identify cells of the visceral endoderm in the A\(^{-/-}\) mice, we stained E6.5 embryos with antibodies to GATA-4, a marker that is expressed in normal visceral endoderm at this stage. The presence of positively staining cells clearly defining the visceral endoderm is seen in the normal embryo (Fig. 2B, panel b, bracket). Although GATA-4 is present in the A\(^{-/-}\) embryo, the abnormal cell morphology and disarray of the visceral endoderm as indicated by GATA-4 staining of the A\(^{-/-}\) embryo is apparent (Fig. 2B, panel a, arrows).

To further characterize the expression of markers of visceral endoderm development, we used ES cells cultured under conditions in which they aggregate to form embryoid bodies. As such, they maintain temporal and spatial relationships in vitro of certain marker proteins expressed in embryos in vivo. Fig. 3A shows RT-PCR analyses of 14-day-old wild-type, A\(^{+/−}\) and A\(^{-/-}\) embryoid bodies for the indicated markers. GATA-4 and apo-E, markers for specification of the visceral endoderm, were
positive in all three genotypes of embryoid bodies. However, some markers for proteins that are secreted from the visceral endoderm and that indicate maturation and function of the visceral endoderm were missing, apo-E, or markedly decreased, AFP (α-fetoprotein), TTR (transthreirin), apo-B, and RBP (retinal-binding protein) in the A-/A- embryoid bodies (Fig. 3A). These results were also confirmed for five of the markers (GATA-4, apo-E, AFP, RBP, and TTR) by real-time PCR analysis (see supplemental Fig. S3 for GATA-4 and RBP).

To further characterize the A-/A- embryoid bodies, we cultured them in suspension on nonadhesive bacteriological culture plates. A marked difference between wild-type and A-/A- embryoid bodies was the continuous shedding of cells from the latter, but not the former, suggesting a defect in cell adhesion in the A-/A- embryoid bodies (Fig. 3B, panels a and b). The embryoid bodies were then transferred to regular tissue culture plates and observed by video time-lapse phase microscopy (Fig. 3B, panels c-f). Wild-type embryoid bodies retained a cohesive rounded morphology throughout the experiments (Fig. 3B, panels c and e), whereas A-/A- embryoid bodies attached to the tissue culture surface within 30 min accompanied by flattening and progressive outward migration of individual cells (Fig. 3B, panels d and f).

The disrupted organization of cell layers in A-/A- embryoids and the shedding of the cells by A-/A- embryoid bodies in suspension and their rapid dispersal on adhesive substrates suggested that absence of NMHC II-A might cause defects in the cell complexes needed for effective cell-cell adhesion. We therefore compared the localization of NMHC II-A and the cell adhesion molecule E-cadherin as well as its intracellular binding partner, β-catenin, in frozen sections of A+/A+ and wild-type E6.5 embryos using immunofluorescence confocal microscopy. As shown in Fig. 4a, NMHC II-A, in addition to being present in the cytoplasm, localized to the contact areas between the cells in wild-type embryos, close to E-cadherin (Fig. 4a, NMHC II-A;...
and d) and $\beta$-catenin (Fig. 5B, panels g and h) at cell-cell interfaces compared with mock-transfected cells. Immunoblot analysis confirms that the decrease in NMHC II-A in these cells is not accompanied by a decrease in E-cadherin and $\beta$-catenin content, consistent with the displacement of these proteins from the cell boundaries (Fig. 5C). In addition, the blots show that there is no change in NMHC II-B and actin. Hence, we demonstrate that the nonmuscle myosin II-A ablation on early embryonic development. A'/? embryos fail to develop past E7.5. The visceral endoderm does not mature molecularly and morphologically. The overall disarray of tissue and cellular organization could be reproduced effectively in vitro using embryoid bodies. A'/? ES cells cannot maintain the typical compact morphology, and cells readily disperse from the embryoid bodies. We also show here that two critical cell-cell adherens junction proteins, E-cadherin and $\beta$-catenin, do not localize normally to cell-cell interfaces both in vivo and in vitro, and tight junction formation is also impaired. It is noteworthy that uvomorulin (cadherin)-deficient ES cells, similar to nonmuscle myosin II-A-deficient cells, cannot aggregate tightly (9) and that $\beta$-catenin-null embryos at E7 showed detachment of cells from the ectodermal cell layer and dispersal into the proamniotic cavity (10).

Myosins I and II are the motors that generate contractility in cells. As described by Krendel and Bonder (11), contractility driven rearrangement of actin bundles can modulate the spatial organization of cell-cell contacts. However, even though nonmuscle myosin II-B is present in both A'/? embryos and A'/? ES cells, this isoform cannot rescue the mislocalization and the subsequent tissue disarray or cell shedding. Indeed, recent work from a number of laboratories (12–14) confirms that these two isoforms have different functions in the same cell and supports a role for myosin II in E-cadherin-mediated adhesions (15). Our data suggest a unique role for myosin II-A during early embryonic development. By exerting tension on actin, which is bound to the cadherin-catenin complex, this myosin plays an essential role in maintaining normal adhesion junctions and cellular organization of the early mouse embryo.

**Acknowledgments**—We acknowledge useful discussions and comments from members of our laboratories and Michael R. Kuehn (NCI) and the expert editorial assistance of Catherine Magruder. We also thank James Sellers and Sachio Kawamoto for critical reading of the manuscript, M. A. C. and R. S. A. especially thank Yvette A. Preston and Antoine Smith for excellent technical assistance.

**REFERENCES**

1. Sellers, J. R. (2000) *Biochem. Biophys. Acta* 1496, 3–22
2. Brennick, A. R. (1999) *Curr. Opin. Cell Biol.* 11, 26–35
3. Golomb, E., Ma, X., Jana, S. S., Preston, Y. A., Kawamoto, S., Shoham, N. G., Goldin, E., Conti, M. A., Sellers, J. R., and Adelstein, R. S. (2004) *J. Biol. Chem.* 279, 2800–2808
4. Takeda, K., Kishi, H., Ma, X., Yu, Z.-X., and Adelstein, R. S. (2003) *Circ. Res.* 93, 330–337
5. Ma, X., Kawamoto, S., Hara, Y., and Adelstein, R. S. (2004) *Mol. Biol. Cell* 15, 2568–2579
6. Martignetti, J. (2002) *Haematologica* 87, 897–898
7. Duncan, S. A., Nagy, A., and Chan, W. (1997) *Development* (Camb.) 124, 279–287
8. Robertson, E. J. (ed) (1987) *Teratocarcinomas and Embryonic stem Cells: A Practical Approach*, pp. 71–112. IRL Press, Oxford
9. Callegaro, A., Samuels, M., Darland, T., Edwards, S. A., Kemler, R., and Adamson, E. D. (1991) *Dev. Biol.* 146, 499–508
10. Haegel, H., Larue, L., Ohnuki, M., Fedorov, L., Herrenknecht, K., and Kemler, R. (1995) *Development* (Camb.) 121, 3529–3537
11. Krendel, M. F., and Bonder, E. M. (1999) *Cell Motil. Cytoskel.* 43, 296–309
12. Lo, C.-M., Buxton, D. B., Chua, G. C. H., Dembo, M., Adelstein, R. S., and Wang, Y.-L. (2004) *Mol. Biol. Cell* 15, 982–990
13. Charterl, P. D., and Wylie, S. R. (2003) *IEEE Proc. Nanobiotecnol.* 150, 111–125
14. Kolega, J. (2003) *Mol. Biol. Cell* 14, 4745–4757
15. Avizyzyte, E., Fincham, V. J., Brungot, V. G., and Frame, M. C. (2004) *Mol. Biol. Cell* 15, 2794–2803
16. Tuille, A. N., Accili, D., Ferrans, V. J., Yu, Z. X., Takeda, K., Grinberg, A., Westphal, H., Preston, Y. A., and Adelstein, R. S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 12407–12412

**Fig. 5.** A, localization of cytoplasmic contractile proteins in wild-type and A'/? cultured ES cells. Immunofluorescence confocal microscopy was used to localize the indicated proteins in wild-type (panels a, c, and e) and A'/? (panels b, d, and f) ES cells. Note that the A'/? colonies have lost the well-defined, rounded shape of wild-type colonies. B, NMHC II-A siRNA-transfected ES cells have defects in localization of E-cadherin (E-Cad) and $\beta$-catenin ($\beta$-Cat). Confocal immunofluorescence microscopy of mock-transfected ES cells (panels a, c, and g) and siRNA-transfected ES cells (panels b, d, f, and h) shows that the siRNA-treated cells have a decreased localization of E-cadherin (panel d compared with panel e) and $\beta$-catenin (panel h compared with panel g) to the cell-cell interfaces. C, an immunoblot confirms that the decrease in NMHC II-A is not accompanied by a decrease in E-cadherin, $\beta$-catenin, NMHC II-B, or actin. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a control for gel loading.

To verify whether NMHC II-A is critical for the formation of other types of cell adhesions, we compared the distribution of NMHC II-A and II-B, actin, ZO-2 (a marker for tight junctions), and connexin-43 (a marker for gap junctions) in wild-type and A'/? cultured ES cells. In wild-type colonies, similar to E6.5 embryos, NMHC II-A localized near plasma membranes and particularly to areas of cell-cell contacts (Fig. 5A, panel a), while NMHC II-B showed more pronounced circumferential staining at the external surface of colonies, with a less pronounced cell-cell border localization (Fig. 5A, panel c). NMHC II-B staining remained prominent at the external surface of the colonies, and its overall distribution was essentially unchanged in A'/? cells, although there was a change in the rounded shape of the colonies (Fig. 5A, panel d). F-actin was localized relatively uniformly to the cell cortex near the plasma membrane in A'/? colonies (Fig. 5A, panel e). In A'/? colonies, total cortical F-actin staining remained relatively high, but a reduction in F-actin staining at the external borders of the colonies was observed (Fig. 5A, panel f). As was found for E-cadherin and $\beta$-catenin, ZO-2 levels, but not connexin 43 levels, were decreased at cell-cell boundaries in A'/? cultured ES cells (see supplemental Fig. S4).

To confirm the above results, we electroporated ES cells with a pool of siRNAs specific for mouse NMHC II-A. Fig. 5B shows that, similar to the A'/? embryos and ES cells, the siRNA-treated cells show a decrease in E-cadherin (Fig. 5B, panels c
Defects in Cell Adhesion and the Visceral Endoderm following Ablation of Nonmuscle Myosin Heavy Chain II-A in Mice
Mary Anne Conti, Sharona Even-Ram, Chengyu Liu, Kenneth M. Yamada and Robert S. Adelstein

J. Biol. Chem. 2004, 279:41263-41266.
doi: 10.1074/jbc.C400352200 originally published online August 2, 2004

Access the most updated version of this article at doi: 10.1074/jbc.C400352200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/08/12/C400352200.DC1

This article cites 15 references, 10 of which can be accessed free at
http://www.jbc.org/content/279/40/41263.full.html#ref-list-1