Unexpected Effects of Wnt-1 on the Differentiation and Adhesion of Myogenic Cells

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Abstract

Wnt proteins are critical for the development of a wide range of organisms. Wnt-1 is expressed by the neural tube and promotes skeletal myogenesis in cultures of intact somites. In other cell types, Wnt-1 enhances cell-cell adhesion mediated by cadherin/catenin complexes. The effect of Wnt-1 on myogenesis was examined in cultures of dissociated epiblast, segmental plan: and somite tissues. Wnt-1 was provided by Rat-2 cells expressing Wnt-1 cDNA. Wnt-1 did not promote myogenesis in prestreak epiblast cultures. In primitive streak stage epiblast cultures, Wnt-1 inhibited myogenesis and the shift from E- to N-cadherin. Both the control and Wnt-1 expressing Rat-2 cells inhibited differentiation of somite and segmental plate cells; however, the inhibition was less for somite cells cultured with Wnt-1. The Rat-2 cells have myogenic potential in that they express desmin, M-cadherin, NCAM, MyoD and MEF-2, but they do not differentiate. They respond to Wnt-1 with an increase in β-catenin, a decrease in cell-cell adhesion and a downregulation of NCAM. The effects of Wnt-1 on adhesion and differentiation depend on intrinsic cell properties that vary with the stage of development. Key words: Wnt-1, cadherin, NCAM, skeletal myogenesis.

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Wnt proteins comprise a family of secreted factors that act locally as signaling molecules during the development of a wide range of organisms from insects to mammals [5, 34, 41, 44]. Wnt-1 is the best studied member of the family and was originally discovered as an oncogene [49, 66]. It was later recognized to be the ortholog of the segment polarity gene wingless in Drosophila melanogaster [60]. Candidate receptors for Wnt proteins have recently been identified as members of the Frizzled family of seven-transmembrane proteins [5, 45, 50]. Wnt signal transduction is thought to occur via an intracellular protein called Disheveled [5, 36] and inhibition of the activity of glycogen synthase kinase 3p (GSK3p) [69]. When active, this serine/threonine kinase helps promotes the degradation of cytoplasmic p-catenin, a mammalian homolog of the Drosophila segment polarity gene product Armadillo [13, 41, 56]. When GSK3p is inactivated as a result of Wnt signaling, its cytoplasmic level of p-catenin rises. p-Catenin is then available to interact with members of the LEF-1/TCF family of transcription factors, translocate to the nucleus, and regulate the transcription of target genes [23,37,48].

p-Catenin also is a critical regulator of cell-cell adhesion via its interaction with the calcium-dependent class of adhesion molecules, the cadherins [24]. The catenins strengthen cell-cell adhesion by linking the cadherins to the actin cytoskeleton [70]. Since Wnt signaling affects p-catenin levels, it is perhaps not surprising that Wnts can affect cell-cell adhesion. In the AtT20 murine neuroendocrine cell line, Wnt-1 expression increased the level of N-cadherin, stabilised its interaction with p-catenin, and strengthened cell-cell adhesion [28]. Wnt-1 also increased the levels of both E-cadherin and plakoglobin, and enhanced cell-cell adhesion in PC12 rat pheochromocytoma cells [4]. Interestingly, Wnt-1-expressing PCI 2 cells no longer differentiated in response to nerve growth factor [51, 61]. Instead, fibroblast growth factor induced neuronal differentiation of the Wnt-1-expressing cells, but not...
Wnt-1 and skeletal myogenesis

The factor(s) responsible for promoting the change in the epiblast that results in their ability to upregulate N-cadherin and form muscle *in vitro* are unknown. Two cytokines that have been implicated in promoting gastrulation, fibroblast growth factor and activin [9, 10, 42, 43, 64, 72], have no affect on cadherin or myosin expression in cultures of preprimitive streak stage epiblast cells [22]. Wnt 5a, 8, and 11 also are present in gastrulating embryos [15, 31, 46]. Although Wnt-1 is expressed somewhat later in development, its effect on myogenesis in prestreak epiblast cultures was tested because Wnt-1 does promote differentiation in the somite and functional redundancy exists among various Wnt family members [2, 11, 15, 17, 31, 38, 46, 47, 55, 58, 63-65]. Furthermore, Wnt-1 has been shown to enhance cell-cell adhesions [4, 28]. Therefore, we tested the ability of Wnt-1 to alter cadherin expression and promote myogenesis in cultures of prestreak epiblast cells.

These studies revealed that Wnt-1-secreting Rat-2 cells did not stimulate myogenesis in cultures of prestreak cells. Surprisingly, they inhibited the differentiation of primitive streak stage epiblast cells. This inhibition of myogenesis was accompanied by a lack of conversion from E- to N-cadherin expression. A slight stimulation of differentiation by Wnt-1 was observed in somite cultures. During the course of these experiments it was discovered that the Rat-2 cells themselves are of the skeletal myogenic lineage, although they fail to differentiate in either the presence or absence of Wnt-1. Thus, it appears that the effects of Wnt(s) on skeletal myogenesis depend on factors intrinsic to the responding cells, most likely reflecting their stage of differentiation and environment.

Materials and Methods

**Rat-2 and Rat-2/Wnt-1 cell cultures**

The original source of the Rat-2 cells was The American Type Culture Collection (ATCC; Rockville, MD). The cells designated Rat-2/Wnt-1 secrete a biologically active Wnt-1 and were generated by infection with the replication-defective retrovirus vector MVVWnt-1 derived from pMV7 [33]. The pMVWnt-1 vector contains mouse Wm-1 cDNA expressed from the viral long terminal repeat, with the bacterial neomycin phosphotransferase gene (neo) expressed from an internal promoter. Control cells designated Rat-2 were infected with the pMV7 vector without the Wnt-1 cDNA. Approximately 50-100 G418-resistant colonies were pooled to generate the infected cell lines used in the experiments. Control and Wnt-1 expressing Rat-2 cells were grown in DMEM containing 10% fetal bovine serum (FBS). G418 (200 μg/ml) was included in the medium for one week after thawing frozen cells but was omitted for the experiments. Two methods known to

wild type cells, indicating that Wnt-1 can simultaneously alter cell-cell adhesion and terminal differentiation. Wnt-1 may also regulate cadherin and catenin expression *in vivo* [62]. When Wnt-1 is deleted in the mouse, the cerebellum and much of the midbrain are missing [40]. Ectopic expression of Wnt-1 in the *Xenopus laevis* embryo results in duplication of the embryonic axis [46].

In addition to their role in the development of the nervous system, heart, kidney, mammary glands, and limb [5, 15, 27, 53-55, 67, 68, 71], members of the Wnt family, including Wnt-1, appear to regulate skeletal myogenesis in embryonic structures called somites. Practically all skeletal muscle arises from precursor cells located in the somites [8]. The initiation of myogenesis within the somite is thought to be stimulated, in part, by Wnt proteins produced by surrounding structures including the neural tube and dorsal ectoderm [2, 11, 15, 17, 31, 38, 47, 55, 58, 64, 65]. Moreover, Wnt-1 promotes myogenesis in cultures of intact somites [47, 64].

The effect of Wnts on myogenesis in the somite appears to be permissive in that some cells already have the potential to differentiate into skeletal muscle prior to their incorporation into the somite [6, 7, 30, 20, 21]. This potential was revealed when the tissue that gives rise to the somites, the segmental plate, was dissociated to produce a single cell suspension and plated at high density in serum free medium. Under these conditions, most cells underwent myogenesis in the absence of the neural tube or notochord [20]. Even the epiblast that gives rise to the segmental plate mesoderm during gastrulation was able to differentiate into muscle *in vitro* after dissociation [21]. Cell-cell interactions that occur within the intact tissue, as well as factors produced from surrounding tissues, repressed myogenesis in epiblast cultures [21].

Skeletal muscle differentiation in cultures of epiblast cells from gastrulating chick embryos is dependent on cell-cell adhesion mediated by N-cadherin [19]. These cells downregulate E-cadherin and upregulate N-cadherin prior to differentiating. This switch in cadherin expression also occurs *in vivo* as epiblast cells ingress through the primitive streak to form the mesoderm [14, 26]. The significance of the upregulation of N-cadherin is demonstrated by the fact that function-perturbing antibodies to N-cadherin, but not E-cadherin, inhibit the expression of sarcomeric myosin in cultured epiblast cells [19]. Furthermore, few epiblast cells from embryos prior to the development of the primitive streak and the initiation of gastrulation switch from E- to N-cadherin or undergo myogenesis *in vitro* even though they express MyoD [19]. Thus, a change occurs within the epiblast at the beginning of gastrulation that enables these cells to express N-cadherin and form muscle in culture.

Materials and Methods

**Rat-2 and Rat-2/Wnt-1 cell cultures**

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stimulate differentiation of skeletal myogenic cell lines such as C2C12 and BHK [59] were applied to the Rat-2 cells. The first method was to grow the Rat-2 and Rat-2/Wnt-1 cells to confluence and then change their medium to DMEM containing 2% horse serum. In the second method, the cells were grown as three dimensional aggregates by suspending 3,000 cells in 20 µl DMEM/10% FBS from the lid of a Petri dish for 24-72 hr and then replating the cells for observation and analysis [59].

**Chick embryo cell cultures**

Embryonated White Leghorn chicken eggs were obtained from Truslow Farms, Inc. (Chestertown MD). Embryos were removed from the yolk and placed in phosphate buffered saline (PBS), pH 7.4. Prior to the development of the primitive streak, embryos were staged by the method of Eyal-Giladi and Kochav [16]. Older embryos were staged according to the method of Hamburger and Hamilton [25].

Epiblast, segmental plate and somite cultures were prepared according to the method of George-Weinstein et al. [20, 21]. Briefly, stages 3-4 epiblasts were isolated from the area opaca, primitive streak and underlying mesoderm and hypoblast. Prestreak epiblasts (stages X-XI) were obtained by removing the hypoblast. The most caudal 8 pairs of somites and segmental plate mesoderm were isolated from stage 13-14 chick embryos. Tissues were dissociated in trypsin and 2.5 X 10^5 cells were placed in 15% of medium composed of Dulbecco's Modified Eagle's medium (DMEM), 5% fetal bovine serum, 5% horse serum (GIBCO/BRL), 5% chick embryo extract, and antibiotics (DES medium). The droplet of cells was placed in the center of a 35-mm tissue culture dish coated with human serum fibronectin and gelatin. Dishes were flooded with 1.5 ml of protein free DMEM/F12 medium 90 minutes after plating. Cultures were incubated at 37°C in 5% CO2 in air.

**Treatment of chick embryo cells with Wnt-1**

Biologically active soluble Wnt proteins are not yet available in purified form. Therefore, cells were treated with Wnt-1 by culturing them in the presence of Rat-2 cells expressing and secreting Wnt-1 (i.e., Rat-2/Wnt-1 cells). Rat-2 cells infected with the vector alone do not produce Wnt-1 and served as a control for the specificity of Wnt-1 activity.

Since Wnt action appears to be short range (autocrine and paracrine), Wnt-1 secreting cells were plated close to the epiblast, segmental plate or somite cells. This was accomplished by mixing chick embryo cells and Wnt-1 secreting cells in various ratios. Cells were plated so that there was a total of 2.5 X 10^4 cells in 15 µl DES medium. Dishes were flooded with 1.5 ml DMEM/F12 medium.

The second method of culturing chick and Wnt-1 secreting rat cells involved plating the chick cells as described previously, then adding two separate drops of rat cells on either side of the spot of chick cells. Rat cells were plated at a density of 5 X 10^3 cells in 5 µl of medium. After attachment, dishes were flooded as described above.

In some experiments, the isolated epiblasts were cultured as an intact epithelium. Pieces of epiblast tissue were allowed to attach to the dish and then 5 X 10^3 nonsecreting or Wnt-1 secreting Rat-2 cells in 10 µl of medium were added to the spot containing the epiblast. Dishes were flooded as described above.

**Immunofluorescence localization**

Immunofluorescence localization of sarcomeric myosin heavy chain, a marker for terminally differentiated skeletal muscle cells, desmin and nuclear proteins was performed as described previously after fixing cells in 2% formaldehyde and permeabilizing with 0.5% Triton X-100 [21]. Localization of cadherins, catenins and NCAM was accomplished by fixing cells in cold methanol. Primary antibodies include MF20 against sarcomeromic myosin heavy chain (Developmental Studies Hybridoma Bank, DSHB) [1], 7D6 to chicken E-cadherin/LCAM (DSHB) [18], 6B3 to chicken N-cadherin [19], 13A9 to N-cadherin [35], anti-M-cadherin (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA), 15B8 to (3-catenin [32], 1G5 to a-catenin [32], anti-E-cadherin (Transduction Laboratories, Lexington, KY), anti-P-cadherin (Transduction Laboratories), rabbit anti-pan-cadherin (Sigma Chemical Co., St. Louis, MO), 5B8 to NCAM (DSHB), 5A5 to the sialylated form of NCAM (DSHB), F5D to myogenin (DSHB), 5.8A to MyoD (a gift from the laboratory of Dr. Harold Weintraub), anti-MEF2 (Santa Cruz), and DE-U-10 to desmin (Sigma). Secondary antibodies included affinity-purified, goat anti-mouse F(ab')2 fragments conjugated with rhodamine or CY3 (Cappel), goat-anti-mouse IgG conjugated to CY3 and goat anti-rabbit IgG conjugated to CY3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). In some experiments nuclei were labeled with a 0.001% solution of bis-Benzamide in PBS. Cells were mounted with elvanol and observed with a Nikon or Zeiss epifluorescence microscope using 40, 60 or 63X objectives and photographed using Kodak TMAX, 400 ASA film or Polaroid type 57 (ASA 3000) film.

**Western immunoblot analysis**

SDS polyacrylamide gel electrophoresis and Western immunoblot analysis were performed as described previously [35]. Primary antibodies included anti-M-cadherin (Santa Cruz), 15B8 to (3-catenin [32], 5B8 to NCAM (DSHB) and 5A5 to the sialylated form of NCAM (DSHB). Secondary antibodies were alkaline.
phosphatase-conjugated goat anti-mouse or rabbit IgG (Fisher Scientific, Pittsburgh, PA) and substrates were NBT/BCIP (Sigma).

Results

Effect of Wnt-1 on myogenesis and cadherin expression in cultures of chick embryo cells

Prestreak epiblast cells

Practically all epiblast cells from primitive streak stage embryos form skeletal muscle in vitro [21]. In contrast, only 1% of epiblast cells cultured from embryos prior to the development of the primitive streak undergo myogenesis [19, 22]. These prestreak epiblast cells do, however, express the skeletal muscle specific transcription factor MyoD, indicating that they have the potential to differentiate [19]. Since Wnt-1 has been shown to promote the differentiation of myogenic cells in the somite, its effect on the differentiation of prestreak epiblast cells was tested by culturing them with Rat-2 cells secreting Wnt-1.

Prestreak epiblast cells (stage X-X1) were randomly mixed in suspension with Rat-2/Wnt-l or control Rat-2 cells. Cultures containing rat cells and epiblast cells in ratios of 1:1 and 1:5 were very dense. The rat and chicken cells were easily distinguished from one another since the epiblast cells were larger and had larger nuclei. The rat cells surrounded small but numerous aggregates of rounded epiblast cells. More aggregates of epiblast cells formed in the presence of the Rat-2/Wnt-l cells than the control Rat-2 cells. This effect is consistent with the reported role of Wnt-1 in enhancing cell-cell adhesion in other cell types [4, 28] and suggested that the chick cells were responding to the Wnt-1.

Neither the Rat-2 nor Rat-2/Wnt-l cells produced an increase in the number of myosin positive avian cells at any of the indicated ratios (Table 1). Therefore, under these conditions, Wnt-1 did not stimulate myogenesis in cultures of prestreak epiblast cells.

Table 1. Effect of Wnt-1 on myogenesis in cultures of preprimitive streak stage epiblast cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Percent Myosin Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epiblast Alone</td>
<td>0 (6)</td>
</tr>
<tr>
<td>Rat-2: Epiblast.</td>
<td>0 (3)</td>
</tr>
<tr>
<td>10:1</td>
<td>0 (3)</td>
</tr>
<tr>
<td>5:1</td>
<td>0 (8)</td>
</tr>
<tr>
<td>1:1</td>
<td>0 (6)</td>
</tr>
<tr>
<td>1:10</td>
<td>0 (3)</td>
</tr>
<tr>
<td>Rat-2/Wnt-l: Epiblast</td>
<td>0 (3)</td>
</tr>
<tr>
<td>10:1</td>
<td>0 (3)</td>
</tr>
<tr>
<td>5:1</td>
<td>0 (3)</td>
</tr>
<tr>
<td>1:1</td>
<td>0 (8)</td>
</tr>
<tr>
<td>1:5</td>
<td>0 (6)</td>
</tr>
<tr>
<td>1:10</td>
<td>0 (3)</td>
</tr>
</tbody>
</table>

Rat-2 or Rat-2/Wnt-l cells were randomly mixed in suspension with prestreak chick embryo epiblast cells (stages X-XI) prior to plating in the indicated ratios. Three days later cells were labeled with the MF20 monoclonal antibody to myosin heavy chain. The number of cultures scored is indicated in parentheses. Greater than 200 cells were scored per culture dish. Wnt-1 did not stimulate myogenesis in cultures of prestreak epiblast cells.

Primitive streak stage epiblast cells

The ability of Wnt-1 to affect the rate of differentiation in cultures of older epiblast cells from gastrulating embryos (stages 3-4) was tested in three ways. First, control Rat-2 cells or Rat-2/Wnt-l cells were mixed in suspension with chick embryo epiblast cells prior to plating. Epiblast cells differentiated to the same extent in the presence or absence of the control Rat-2 cells (Table 2; Fig. 1). Unexpectedly, when the epiblast cells were cultured with Rat-2/Wnt-l cells, there was a dramatic decrease in the percentage of myosin positive cells compared to controls (Table 2; Fig. 1). Therefore, Wnt-1 either directly or indirectly inhibited skeletal muscle differentiation.

The second method for co-culturing the rat and epiblast cells was to plate them as side-by-side spots of high cell density. Consistent with the results obtained with randomly mixed cells, Wnt-1 secreting Rat-2 cells reduced the percentage of myosin positive epiblast cells (Fig. 1). The extent of inhibition depended on the proximity of the Rat-2/Wnt-l cells to epiblast cells; the closer the spots were to each other the greater the reduction in myosin positive cells. The further the epiblast cells were from the Rat-2/Wnt-l cells, the more myosin positive cells were observed. In contrast, muscle
cells were scattered throughout the spot of epiblast cells cultured beside control Rat-2 cells. These results provide strong evidence that the inhibition of epiblast myogenesis was due to the production of Wnt-1 since Wnt proteins are known to be sequestered by cell surfaces and the extracellular matrix and may not diffuse over long distances [3, 33, 52].

Although Wnt-1 inhibited myogenesis in cultures of dissociated epiblasts, it was possible that these cells would respond differently to this molecule if they remained in contact with their original neighbors. Therefore, the effect of Wnt-1 on myogenesis was tested in cultures of intact epiblast tissue. A few groups of myosin positive cells were present in 48 hour cultures of epiblast tissue plated alone or in the presence of control Rat-2 cells (Fig. 1). In contrast, no myosin positive cells were observed in cultures containing Wnt-1 secreting Rat-2 cells (Fig.1). Therefore, Wnt-1 had a similar effect in cultures of intact epiblast tissue and dissociated epiblasts: myogenesis was inhibited.

Cadherin expression in cultures of primitive streak stage epiblast cells

Wnt-1 expression has been shown to stabilize cell-cell adhesion mediated by cadherin proteins [4, 28]. Epiblast cells downregulate E-cadherin and upregulate N-cadherin when they enter the primitive streak in vivo and also when they are placed in culture [19]. This switch in cadherin expression is required for cultured epiblast cells to undergo skeletal myogenesis [19]. Since Wnt-1 inhibited muscle differentiation in epiblast cultures and is known to stabilize cell-cell adhesion, it was possible that E-cadherin mediated adhesions were stabilized and the switch to N-cadherin was prevented. Therefore, immunofluorescence localization was performed to analyze the distribution of N- and E-cadherin in epiblast cells cultured in the presence of Wnt-1 secreting and control Rat-2 cells.

The percentages of myosin, N-cadherin and H-cadherin positive cells were similar in cultures containing epiblast cells alone or a 1:5 mixture of control Rat-2 cells and epiblast cells (Table 3). In contrast, culturing epiblast cells with Rat-2/Wnt-1 cells resulted in significant differences in the expression of all three molecules compared to controls; N-cadherin and myosin were decreased while the number of cells with detectable E-cadherin increased (Table 3). Thus, the Wnt-1 induced inhibition of myogenesis in epiblast cultures correlates with a decrease in the switch from E- to N-cadherin expression that normally precedes differentiation.

Figure 1. Effect of Wnt-1 on myogenesis in epiblast cultures. Primitive streak stage epiblast cells (EPI) were cultured in the presence of control Rat-2 cells (RAT/EPI) or Wnt-1 secreting Rat-2 cells (Wnt/EPI). Epiblasts were dissociated and either mixed in suspension with the Rat-2 cells before plating (A-C) or the two cell types were cultured as side-by-side spots (D-F). Epiblasts were also plated as intact tissues (G and H). Cells were stained with the MF20 monoclonal antibody lo sarcomeric myosin heavy chain and a secondary antibody conjugated with rhodamine. Wnt-1 secreting cells inhibited the expression of myosin in epiblast cells when the cell types were mixed prior to plating (C), cultured as side-by-side spots (E), or when the epiblast was plated as an intact epithelium (H). In side-by-side cultures, myosin positive epiblast cells were observed at a distance from the Wnt-1 secreting cells (F). The control Rat-2 cells had no effect on differentiation (B, D, and G). Bar = 28 µm in A-C and 42 urn in D-H.

Somite and segmental plate mesoderm cells

The inhibitory effect of Wnt-1 on epiblast myogenesis was opposite to the positive effect of Wnt-1 reported for newly formed somites [47, 64]. The difference in these results could be attributable to the fact that epiblast cells are developmentally younger than mesoderm cells. Alternatively, the stimulatory effect of Wnt-1 observed in somite cultures may reflect the fact that the somites were cultured as intact tissues instead of dissociating them prior to plating. Therefore, the effect of Wnt-1 on myogenesis was tested in cultures prepared from a single cell suspension of somite cells.
Table 3. Effects of Wnt-1 on the expression of myosin and cadherins in cultures of primitive streak stage epiblast cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Percent Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myosin</td>
</tr>
<tr>
<td>Kpiblast alone</td>
<td>34 ± 5 (11)</td>
</tr>
<tr>
<td>Rat-2 and Kpiblast</td>
<td>34 ± 8 (12)</td>
</tr>
<tr>
<td>Rat-2/WntM and Epiblast</td>
<td>8 ± 5 (13)</td>
</tr>
</tbody>
</table>

Epiblast cells were mixed in a ratio of 1:5 with control Rat-2 cells or Rat-2/Wnt-1 cells. Two days after plating cells were labeled with monoclonal antibodies to sarcomeric myosin heavy chain, N-cadherin, or E-cadherin. Results are the mean ± standard deviation. The number of cultures scored is indicated in parentheses. Greater than 200 cells were scored per dish. Culturing epiblast cells with Rat-2/Wnt-1 cells resulted in a decrease in the percentages of myosin and N-cadherin positive cells (p < 0.005 and 0.0005, respectively). The percentage of E-cadherin positive cells was higher in the presence of Wnt-1 (p < 0.005).

Table 4. Effects of Wnt-1 on myogenesis in cultures of segmental plate and somite cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Percent Myosin Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segmental Plate (SP) Alone</td>
<td>51 ± 12 (8)</td>
</tr>
<tr>
<td>Rat-2 and SP</td>
<td>23 ± 8 (6)</td>
</tr>
<tr>
<td>Rat-2/WntM and SP</td>
<td>15 ± 9 (5)</td>
</tr>
<tr>
<td>Somite Alone</td>
<td>46 ± 17 (4)</td>
</tr>
<tr>
<td>Rat-2 and Somite</td>
<td>17 ± 4 (5)</td>
</tr>
<tr>
<td>Rat-2/Wnt-l and Somite</td>
<td>30 ± 5 (5)</td>
</tr>
</tbody>
</table>

Nonsecreting or Wnt-1 secreting Rat-2 cells were plated in separate spots alongside of somite or segmental plate (SP) cells. Twenty-four hours later, cells were labeled with the MF20 monoclonal antibody to myosin heavy chain. Results are the mean ± standard deviation. Greater than 200 cells were scored per culture dish. The number of cultures scored is indicated in parentheses. The differentiation of somite cells was inhibited by both nonsecreting (p < 0.005) and Wnt-1 Rat-2 cells (p < 0.05), although the inhibition by the Rat-2/Wnt-l cells was less than that produced by the Rat-2 cells. Both types of Rat-2 cells also inhibited myogenesis in segmental plate cultures (p < 0.005).

Effect of Wnt-1 on Rat-2 cells

Responsiveness of Rat-2 cells to Wnt-1

While investigating the effects of Wnt-1 on chick embryo cells, several interesting properties of the Rat-2 cells themselves were revealed that demonstrated their responsiveness to Wnt-1. First, if the cells were allowed to become post-confluent, a consistent difference was noted in the pH of the conditioned medium between the parent Rat-2 cell line and those secreting Wnt-1. The medium of Rat-2 cultures was consistently more acidic than that of Rat-2/Wnt-1 cultures, e.g., pH 6.9 for Rat-2 cells and pH 7.2 for Rat-2/Wnt-1 cells. This difference suggested that Wnt-1 induced a change in glucose metabolism and indicated that the Rat-2 cells respond in some way to Wnt-1. The Rat-2 cells also responded to Wnt-1 with an increased level of cytoplasmic P-catenin as determined by immunofluorescence localization (Fig. 2, panels A and B) and Western immunoblotting (not shown; M. Giarre and A. Brown, unpublished). The presence of P-catenin at the plasma membrane in immunofluorescence stainings suggested that the Rat-2 cells expressed a cadherin. This notion was further strengthened when a pan-cadherin antibody stained the plasma membrane. However, neither N-, P-, nor E-cadherin was detected in either Rat-2 or Rat-2/Wnt-1 cells using specific antibodies. Instead, M-cadherin was present at cell-cell contact sites in both control and Wnt-1 expressing cells (Fig. 2D). No significant difference in the level of M-cadherin in the two rat cell lines was
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Figure 2. Immunofluorescence localization of β-catenin, desmin, M-cadherin, MyoD, and MEF-2 in Rat-2 i-c/A. Rat-2 cells (panels A, C, D-F) and Rat2/Wnt-l (panel B) cells were grown on glass cover slips, fixed with either methanol (A, B, D) or paraformaldehyde followed by detergent permeabilization (C, E, F), and stained with antibodies to β-catenin (A, B), desmin (C), M-cadherin (D), MyoD (E), or MEF-2 (F). Cells in panels A-C and D-F were photographed using a 40X and 63X objective, respectively. Note the increase in cytoplasmic staining for β-catenin in the Rat-2/Wnt-l cells (B) compared to the control Rat-2 cells (A).

Myogenic potential of Rat-2 cells

The presence of M-cadherin suggested that the Rat-2 cell line was of skeletal muscle lineage [12]. This was confirmed by the presence of desmin and two transcription factors, MEF2 and the skeletal muscle specific transcription factor MyoD (Fig. 2. panels C, E and F). However, neither the control nor the Wnt-l secreting Rat-2 cells expressed myogenin, another skeletal muscle specific transcription factor, nor did they express sarcomeric myosin. Experimental manipulations known to promote skeletal muscle differentiation of other cell lines, including replacing the 10% fetal bovine serum in the medium with 2% horse serum or culturing the cells in suspension [59], did not induce the expression of myogenin or sarcomeric myosin in either cell line. Thus, although the Rat-2 cells appear to have myogenic potential, they fail to differentiate, even in the presence of Wnt-l which promotes differentiation of somite cells.

Cell-cell adhesion of Rat-2 cells

Interestingly, although the Rat-2 cells express a cadherin and the levels of β-catenin were increased in response to Wnt-l, the Rat-2/Wnt-l cells did not adhere to one another as tightly as the control Rat-2 cells. This was readily apparent when the cells were cultured in suspension as a strategy to induce their differentiation. Both cell types formed aggregates; however, when the cells were collected by pipet after 24-48 hr and replated onto tissue culture dishes, the Rat-2/Wnt-l cells dispersed into multiple smaller aggregates, whereas the Rat-2 cells remained as a single, large aggregate (Fig. 4). This was not due to a decrease in viability as essentially all cells re-attached and continued to grow.

Since the level of M-cadherin was similar in the two cell types, we examined the expression of NCAM, another adhesion protein present in developing skeletal muscle [39]. Indeed, the Rat-2 cells expressed a nonpolysialylated 145 kD isoform of NCAM. However, both immunoblot analysis (Fig. 3) and immunofluorescence light microscopy (not shown) revealed that NCAM expression was downregulated in the Rat-2/Wnt-l cells, most likely explaining the reduced strength of cell-cell adhesion among the Rat-2/Wnt-l compared to control cells. A similar downregulation of NCAM by Wnt-1 has been observed in PC12 cells (R. Bradley and A. Brown, unpublished observation).

Discussion

Wnt proteins are critical for embryonic development [5, 44]. Signaling by Wnt-l and other family members is
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Figure 4. Cell-cell association of Rat-2 and Rat-2/Wnt-1 cells. Rat-2 (A) and Rat-2/Wnt-1 (B) cells were cultured as a three dimensional aggregate in suspension as described in the Materials and Methods and by Redfield et al. (1997). After 24 hr the aggregates were collected by pipet, placed in culture dishes and visualized by phase contrast microscopy. Whereas the control Rat-2 cells remained as a single large aggregate, the Rat-2/Wnt-1 cells dispersed into multiple smaller aggregates. This decrease in the strength of cell-cell adhesion was obvious and consistent, and was seen with Rat-2 cells from two independent infections for Wnt-1 expression.

thought to alter transcription by increasing the level of cytoplasmic [3-catenin which forms complexes with transcription factors of the HMO family [23, 41, 48]. In addition, Wnt-1 also affects cell-cell adhesion [24]. For this reason, it is perhaps not surprising that Wnt-1 can affect skeletal muscle differentiation. Wnt proteins produced in the neural tube and dorsal ectoderm have been implicated in the stimulation of myogenesis in the somite [2, 11, 15, 17, 31, 38, 47, 55, 58, 64, 65]. Moreover, Wnt-1 promotes myogenesis in cultures of intact somites [47, 64].

The effects of Wnt-1 on muscle differentiation have been difficult to assess in vitro due to the fact that purified Wnt-1 is not available and must instead be provided by stably transfected cell lines. In our experiments, both the parent Rat-2 cells and Wnt-1 expressing Rat-2 cells inhibited myosin expression in cultures of dissociated somite or segmental plate tissue. A similar inhibitory effect of Rat-2 cells on skeletal muscle differentiation was previously observed in cultures of intact somites [64] and may be due to growth factors secreted by the rat cells to which the segmental plate and somite cells are capable of responding. The reduction in differentiation in cultures of intact or dissociated somites containing Rat-2/Wnt-1 cells was less than that observed in cultures with control Rat-2 cells. This suggests that Wnt-1 may override the inhibition to some degree and stimulate differentiation, an effect consistent with its proposed role in vivo.

In contrast, Wnt-1 inhibited skeletal muscle differentiation in primitive streak stage epiblast cultures. The Wnt-1 induced decrease in epiblast differentiation most likely results from its effect on events that regulate cadherin expression. The switch from E- to N-cadherin that is required for myogenesis in epiblast cultures was inhibited in the presence of Wnt-1. The importance of the shift from E- to N-cadherin for myogenesis is supported by the fact that the antibodies to N- but not E-cadherin block the differentiation of primitive streak stage epiblast cells [19]. Prestreak stage epiblast, which do not differentiate in vitro, fail to downregulate E-cadherin and upregulate N-cadherin [19]. Wnt-1 maintains the expression of E-cadherin in epiblast cells in vitro, and therefore, is promoting the cell-cell contacts that occur within the epithelium of the epiblast in vivo.

Skeletal muscle cells express several cadherins, including N-, M-, and cadherin-11, but not E-cadherin [39]. Although N-cadherin appears to be the primary cell-cell adhesion molecule for promoting myogenesis [19], somite cells from N-cadherin null mice are able to differentiate and appear to express another cadherin [57]. Moreover, either N- or E-cadherin support MyoD expression in cells from Xenopus leavis embryos [29] and in chick embryo epiblast cells [19]. In addition, BHK cells expressing exogenous E-cadherin can be induced to differentiate into skeletal muscle [59]. Therefore, although the expression of E-cadherin appears to be incompatible with epiblast differentiation [19], it is not likely to be a direct inhibitor of this process. Instead, we favor the hypothesis that the downregulation of E-cadherin which occurs as epiblast cells enter the primitive streak or when they are dispersed and placed in culture, is the hallmark of multiple changes occurring in these cells that results in a more permissive internal environment for the expression of muscle genes. In this regard, the inhibition of epiblast differentiation by Wnt-1 would result more from a general block in the switching of developmental programs than a targeted effect on E-cadherin alone.

The difference in the response of primitive streak stage epiblast and somite cells to Wnt-1 may reflect their stage of development and the type of cadherin they express. Wnt-1 prevented the conversion from E- to N-cadherin in epiblast cells, whereas somite cells already express N-cadherin. Therefore, the effects of Wnt-1 appear to depend on the position of the cells within the myogenic pathway. Under these conditions, Wnt-1 blocks epiblast cells from becoming mesodermal, but after they have undergone this transition. Wnt-1 may facilitate their progression towards differentiation.

During these studies we noted that the control and Wnt-1-expressing Rat-2 cells express nuclear MyoD. These cells also express MEF2, desmin, NCAM, and M-cadherin, all of which are expressed by skeletal muscle cells. However, neither the control nor Wnt-1 expressing Rat-2 cells could be induced to differentiate by methods used to stimulate differentiation of other muscle cell
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lines, including scrum reduction or suspension culture. This may reflect the fact that the Rat-2 cells continue to divide under these conditions and therefore may have internal signals that oppose withdrawal from the cell cycle and differentiation.

Although the Rat-2 cells were not induced to differentiate by Wnt-1, they did respond to the Wnt-1 with an increase in their level of cytoplasmic $\beta$-catenin. Surprisingly, the Wnt-1 expressing Rat-2 cells exhibited decreased cell-cell adhesion compared to control cells. This is in contrast to reports that Wnt-1 expression enhances cell-cell adhesion in other cell lines by increasing the stability of cadherin/catenin complexes [4, 28]. However, no significant difference in the expression of M-cadherin was detected in response to Wnt-1. Instead, the level of NCAM was reduced, likely explaining the decrease in cell-cell adhesion.

Wnt proteins appear to play important roles in regulating the maturation and differentiation of muscle precursors and the morphogenesis of muscle. These processes may involve the effects of Wnt(s) on both cell adhesion and gene transcription. The end result of Wnt signaling, however, is likely to depend on the specific Wnt(s) the cells are exposed to, the Wnt receptors they express, and on the intrinsic properties of the responding cells. Intrinsic properties that are expected to affect Wnt signaling include the type of adhesion molecules and the transcription factors the cells express, as well as their mechanisms for regulating cell cycle progression, properties that vary with their stage of development and local environment.

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References

[12] Donalies M, Cramer M, Ringwald M, Starzinski-Powitz A: Expression of M-cadherin, a member of the cadherin multigene family, correlates with
Wnt-1 and skeletal myogenesis


[38] Marcelle C, Stark MR and Bronner-Fraser M: Coordinate actions of BMPs, Wnts, Shh and
Wnt-1 and skeletal myogenesis


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