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Short Communication

Correlation between the expression of the HNK-1 epitope and cellular invasiveness in prestreak epiblast cells of chick embryos

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ABSTRACT During avian gastrulation, certain cells present in the epiblast layer ingress through the basement membrane sealing the basal surface of themselves. Previously we reported that chick prestreak epiblast cells show two different behavioral phenotypes upon reconstituted basement membrane and laminin gel *in vitro*. Half of the dissociated epiblast cells invade the gel substratum after one-day of culture, whereas the others attach to the gel but do not invade. It is expected that such heterogeneity in the behavior of the epiblast cells reflects some mechanism that sorts the cells into those that will ingress into the blastocoelic cavity and those that will remain in the epiblast layer. To test this hypothesis, we dissociated chick prestreak epiblast cells into single cells, cultured them on the laminin gel, and then stained them with anti-HNK-1 antibody. This antibody binds to an epitope present on half of the prestreak epiblast cells which are thought to differentiate into presumptive mesoendodermal cells. We found that 80% of the invasive epiblast cells were HNK-1-positive whereas 77% of the non-invasive cells were HNK-1 negative. In the case of invasive cells, the edges of the proteolytic holes made by the invasive cells were often stained. These results suggest that the cells expressing the HNK-1 carbohydrate chain are preferentially invasive, and this induces selective ingression of the carrier cells for mesoendodermal differentiation *in vivo*.

KEY WORDS: *chick prestreak epiblast, HNK-1 epitope, invasion, germ layer, subpopulation.*

In chick embryos, all of the early somatic cells are derived from a columnar epithelium called the epiblast. At the primitive streak (PS) stage, some of the epiblast cells ingress through the PS to form the mesoderm and endoderm. The other cells remaining in the epiblast layer form ectodermal tissues. The mechanism that results in this ingression of specific cells from the epiblast layer remains to be determined (Bellairs, 1986; Harrisson *et al.*, 1991; Toyoizumi and Takeuchi, 1995; Toyoizumi *et al.*, 1997).

Investigation of this phenomenon may allow the identification of the mechanism of divergence of ectodermal and mesoendodermal cell allocation and differentiation (Bellairs, 1986; Harrisson, 1989; Sanders and Prasad, 1989; Stern and Canning, 1990; Watt *et al.*, 1993; Tam *et al.*, 1997). The epiblast develops a mature basement membrane (BM) late in the prestreak stage before the onset of ingression (Duband and Thiery, 1982; Sanders, 1982; Harrisson and Vakaet, 1989; Zagris and Chung, 1990; Harrisson, 1993). Therefore, ingressive cells need to penetrate the barrier of the continuous BM undersealing the epiblast layer. In order to determine when epiblast cells acquire this ability, we dissociated prestreak epiblast cells at various stages of development, and seeded them at low density on the laminin gel to avoid cell-to-cell communication (Toyoizumi and Takeuchi, 1995). We found that half of the late prestreak-stage (stage XII-XIII) epiblast cells invaded the laminin gel whereas the remainder did not invade but simply attached to the gel substratum. Similar phenomenon was also observed when we used reconstituted BM instead of the laminin gel (Toyoizumi *et al.*, 1997). This suggests that, the ability to invade the BM for ingression is equipped in half of the late prestreak epiblast cells.

Meanwhile, Canning and Stern (1988) found that the expression of the HNK-1 carbohydrate antigen (Human Natural Killer-1; Leu-7 antigen or CD57) had a mosaic pattern in chick prestreak epiblast cells. They reported that approximately 35% of the late prestreak epiblast cells express HNK-1 epitope. In chick embryos at the early PS stage, HNK-1 antigen is expressed in mesoderm and endoderm cells, but is not expressed in the epiblast. HNK-1 expression is transient, because after gastrulation is completed, its expression rapidly ceases in all germ layers. In avian somite stage embryos, the emigrating neural crest cells express HNK-1 antigen and the HNK-1 antibody perturbs the migration of the neural crest cells (Bronner-Fraser, 1987). From these studies, it is expected that HNK-1 expression on the prestreak epiblast cells plays an important role in their ingression during gastrulation. Furthermore, Stern and Canning (1990) proposed that, among prestreak epiblast cells, only the subpopulation that differentiates into the mesoendodermal lineage

Abbreviations used in this paper: BM, basement membrane; PS, primitive streak.

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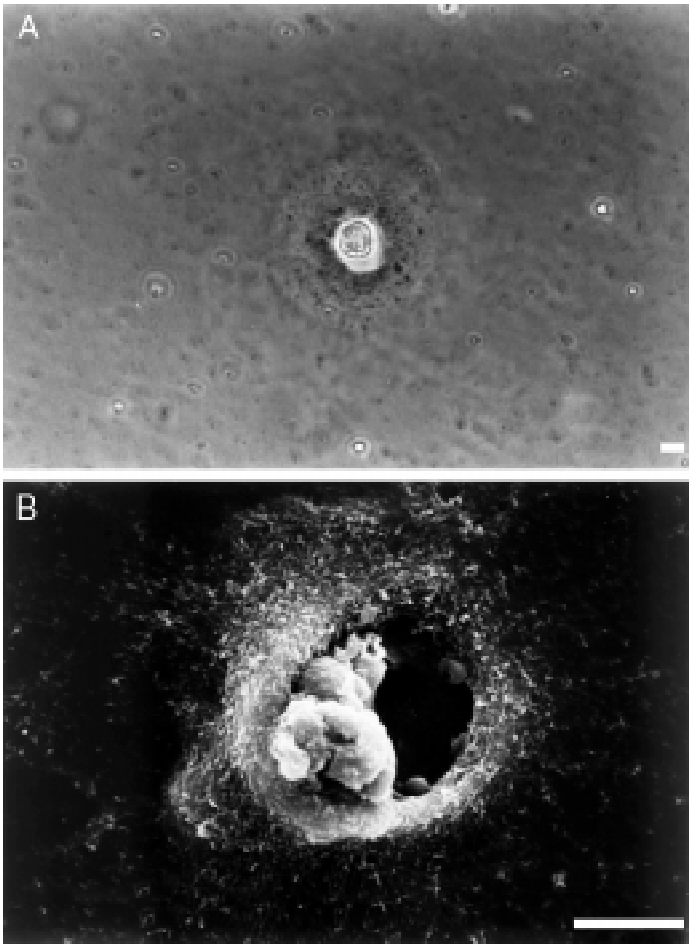

Fig. 1. Invasion of the prestreak epiblast cells *in vitro*. **(A)** *Phase contrast micrograph of a prestreak-stage epiblast cell on the laminin gel after 24 hours of culture. Note that the cell is encircled by black ring-like marks.* **(B)** *Scanning electron micrograph of a stage XIII epiblast cell on the gel substratum. Note that the cell has dissolved the gel to make a hole and still adheres to the wall of the hole. Bars, 10 μm.*

expresses the HNK-1 antigen, based on their preferential extirpation of HNK-1 positive cells using complement-conjugated HNK-1 antibody. If the heterogeneity in the ability of epiblast cells to invade functions as a "cell sorter" to separate presumptive mesoendodermal cells (ingressive cells) from presumptive ectodermal cells (non-ingressive cells), then the invasive cells should express the HNK-1 epitope as occurs exclusively in early mesoendodermal cells.

With this background in mind, we applied HNK-1 immunostaining to single prestreak epiblast cells that had been cultured on the laminin gel for 24 hours. After a few hours of culture, almost all of the dissociated prestreak epiblast cells had attached to the substratum in spherical forms. After 24 hours in culture, half of the cells were encircled by black ring-like marks when observed with phase contrast microscopy (Fig. 1a), as was previously reported (Toyoizumi and Takeuchi, 1995). Scanning electron microscopy revealed that the encircled cells had dissolved the laminin gel beneath them by making proteolytic holes (Fig. 1b). Thus, the ring-like marks were judged to be the edges of such holes. Eventually, the cells adhered to the walls or bottoms of these holes. Thus we could detect the invasive cells by the characteristic ring-like marks in phase-contrast microscopy. After 24 hours of culture on the laminin gel, 41% of stage XII-XIII cells (584/1435) were counted as invasive.

These results imply that these individual prestreak epiblast cells have acquired their invasiveness. In all experiments using single cell culture, some cells invaded the gel, whereas the others did not. We applied monoclonal anti-HNK-1 antibody to the gel substratum on which these two populations of epiblast cells had been cultured for 24 hours and used a secondary antibody that was labeled with colloidal gold (total cell number=1595). Silver enhancement of the colloidal gold staining was used after the immunostaining.

We found that many of the invasive cells were stained with the colloidal gold whereas most of the non-invasive cells were not stained (Fig. 2). The edges of the holes made by the invasive action of the cells (ring-like structures) were also stained with colloidal gold. This may be due to the transfer of the HNK-1 carbohydrate antigen on the cell surface to the gel substratum during the invasive downward movement. Two or five μl of stromelysin (MMP-3; 0.5 unit/ml), an enzyme that can lyse laminin, was dropped onto the air-dried laminin gel, and incubated for 40-60 min at 37ºC. The dry gel was then rehydrated, further incubated with our culture medium for one day, and then stained with anti-HNK-1 antibody. We confirmed that the gel was digested in a circular form by the enzyme solution, however, the characteristic, ring-like HNK-1 staining was not observed.

Figure 3 summarizes the correlation between the invasive behavior and the expression of the HNK-1 epitope for individual cells. Staining patterns of the HNK-1 positive/negative cells were classified into four groups. In summary, 80% of the invasive cells or their holes were stained intensely with HNK-1 antibody (548/686), whereas, 77% of the non-invasive epiblast cells were HNK-1 negative (703/909). In the control samples where the first antibody was not used, neither the invading cells nor the ring-like structures were stained (total cell number=530). The proportion of the HNK-1 positive cells was about the same in stage XIII prestreak cells that had been in culture for less than 90 min (42%, n=1296) as those that had been in culture for 24 hours (47%). Thus, we believe that HNK-1 expression was consistently maintained in the same cells.

In conclusion, there is a strong correlation between the expression of HNK-1 and the invasiveness in the prestreak epiblast cells ($P<0.001$, $\chi^2=513.6$; tested by 2x2 contingency table test).

Our experiments revealed that HNK-1 positive prestreak epiblast cells have the ability to invade after dissociation and culture on the laminin substratum. The ability of these cells to invade the substratum seems to be free of the community effect of interaction with neighboring cells, because we dispersed the prestreak epiblast tissue into individual cells and seeded them at a very low density. This suggests that the specific interaction between the cells carrying the HNK-1 carbohydrate chain and the substratum plays a key role in the preferential invasion of these cells as opposed to the other prestreak cells without HNK-1. It has been reported that the HNK-1 moiety present on the cell surface could bind directly to the G2 domain of laminin (Hall *et al.*, 1995; Hall *et al.*, 1997). This implies the possibility that a specific signaling pathway for invasion can be activated via direct coupling of the HNK-1 antigen molecule(s) and laminin. This pathway would not be activated in HNK-1 negative cells. Further evidence for this idea comes from the finding that highly invasive or metastatic cell lines express the HNK-1 antigen at levels higher than less invasive cell lines derived from the same tissues (Ring and Addis, 1986; Mooy *et al.*, 1995).

The HNK-1 epitope is known to be present on the surface of various cell adhesion molecules including N-CAM, MAG, Ng-CAM, and integrins (Schachner, 1989; Lallier and Bronner-Fraser, 1991). The HNK-1 epitope has been shown to be a sulfated trisaccharide (HSO3-GlcAβ1-3Galβ1-4GlucNAc), and the strong negative charge of the sulfated base has been proposed to regulate the activity of cell-cell or cell-substratum adhesion molecules resulting in changes of cell shape and behavior. Terayama *et al.,* (1997) revealed that transfection of the glucuronyltransferase cDNA into COS-1 cells not only induced expression of the HNK-1 epitope on the cell surface but also resulted in the formation of many long cellular processes leading to marked morphological changes of the cells. These changes were most prominent on the laminin substratum. In the case of embryonic cells, HNK-1 expression has also been shown to be critical for their behavior. Sanders and Cheuing (1988) reported that HNK-1 antigen is used as a scaffold for neural crest migration and neurite extension. Based on these studies, we propose that the HNK-1 chain on the surface of the carrier cells induces their invasive vertical migration (i.e., ingression) across the laminin-rich BM into the blastocoelic cavity *in vivo*. As a result, after ingression into the cavity, the HNK-1 positive cells will be open to receive the inducing signal for mesoendodermal differentiation more than the negative cells remaining in the epiblast sheet. This will lead to the stochastic preference of HNK-1 positive cells for mesoendodermal lineage rather than ectodermal one.

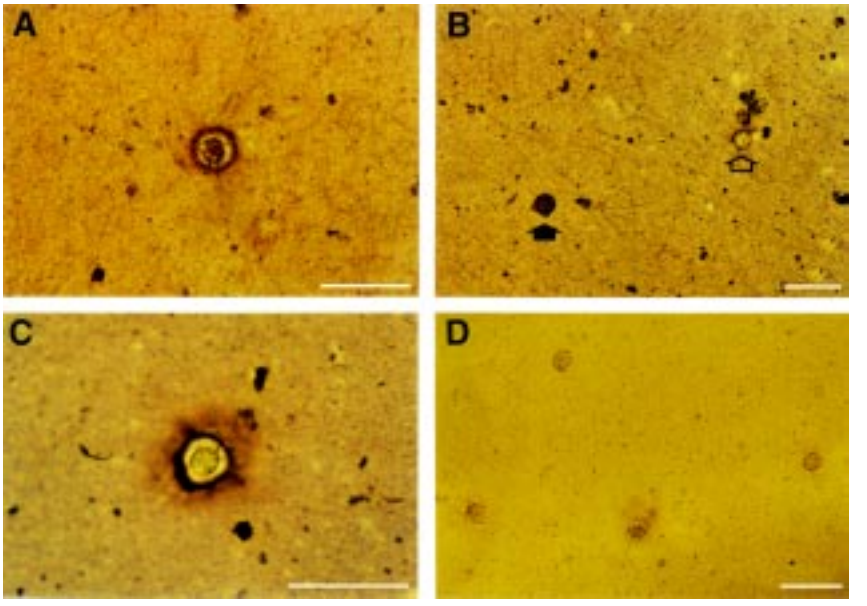

Fig. 2. Anti-HNK-1 immunostaining of stage XIII epiblast cells after 24 hours of culture on the laminin gel. **(A)** *An invading single cell that is stained on both the cell surface and the edge of the hole made during invasion.* **(B)** *Non-invasive cells. The left cell (arrow) is HNK-1-positive, while the right cell (open arrow) is HNK-1-negative.* **(C)** *The stained edge of the hole made by an invading single cell. With some of the invading cells, there was little staining of the cell bodies, though the edges of the invasive holes were very intensely stained as in this case.* **(D)** *Cells in the control experiment without the first antibody, which are not stained. Bars, 50 μm*

We should be careful in discussing the HNK-1 expression in the context of mesoendoderm induction. Cooke (1993) reported that spreading response like mesodermal cells can be induced in epiblast cells by administration of activin or bFGF, both of which are regarded to be endogenous mesoderm inducers in chick embryogenesis. However, despite the behavioral transformation of most of epiblast cells by activin or bFGF, the proportion of HNK-1 positive cells is not altered (Cooke, 1993). Furthermore, Cooke *et al.* (1994) reported that there was no change in the local background incidence of epiblastic HNK-1 expression in ectopic streaks induced by activin-secreting grafts. These studies suggest that the HNK-1 expression is *not* an absolute requirement for mesoendoderm induction. Based on our results, we can at least say that the gathering and aggregation of HNK-1 positive cells along the initial PS, which is observed in normal embryos, may not be a prerequisite for induction of ingression movement across the BM. In order to determine the physiological role of the HNK-1 chain in cell movement before/during gastrulation, we need to study the expression mechanisms of both glucuronyltransferase/sulfotransferases, involved in the biosynthesis of the HNK-1 moiety, and gelatinases (matrix metalloproteases) associated with invasion, in the chick early embryo.

Experimental Procedures

Cell culture

Chick embryos at stage XII-XIII of Eyal-Giladi & Kochav's normal developmental table (1976) obtained by 4-6 hours of incubation were used. We used the eggs of Boris Brown strain in all the experiments. Stage XII epiblast cells were dissected from the slightly caudal region of the *area pellucida*. In the case of stage XII embryos, we could identify the posterior side of the blastoderm with the expanding secondary hypoblast underlying the posterior part of the epiblast (see Mogi *et al.*, 1998). Stage XIII epiblast cells were dissected from the center of the *area pellucida*.

The epiblast cells were dissected and collected with a pair of glass needles according to procedures described previously (Toyoizumi and Takeuchi, 1995). The fragments were then transferred into a well of 96 well test plate (Falcon Co., '3072') filled with Ca^{2+}-free Spinner's Minimum Essential Medium (S-MEM; Gibco Co.). After immersing

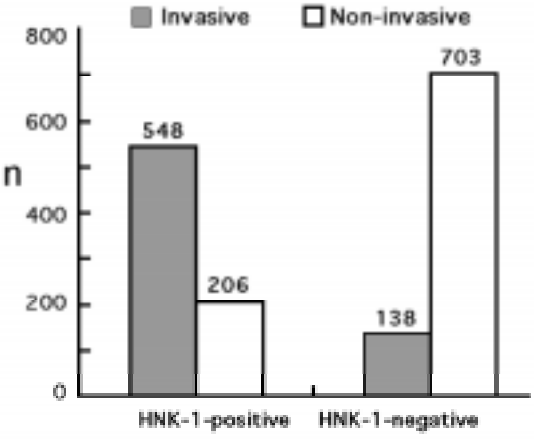

Fig. 3. Correlation between invasiveness and HNK-1 expression in the prestreak (stage XIII) epiblast cells. *The Y-axis represents the number of cells examined that fell into each group (n=1595 in total). Note that most of the invasive cells carried the HNK-1 epitope, whereas most of the non-invasive cells did not.*

the epiblast cells in S-MEM for 10 minutes, the fragments were dissociated into single cells by pipetting 15-20 times gently. The cells were again immersed in S-MEM for 10 minutes.

The dissociated single cells were seeded onto the gel substratum for the invasion assay. In order to make laminin gel substratum, 30-50 μl of the turbid laminin solution (1 mg/ml; BT-267, Biomedical Technologies Inc. Co.) was mounted on the bottom of plastic Petri dishes (35 mm in diameter, Falcon Co., '3001') and incubated at 37ºC for several hours. This product contains more than 95% laminin (information form the company). The gel was rinsed once with the medium to eliminate harmful solvents prior to the assays. Three ml of RPMI1640 medium (Gibco Co.) supplemented with 2% chick serum (Cosmo Bio Co.) was poured into the plastic Petri dish coated with the gel substratum. The dissociated epiblast cells were seeded onto the laminin gel with a Pipetman and cultured in a humid atmosphere containing 5% CO2-95% air at 37ºC for 24 hours. Thereafter, we observed the behavior of the epiblast cells with a phase-contrast microscope, and counted the total cell number attached to the gel and the number of the cells which made a hole in the gel for each dish. Then we calculated the percentage of the invasive cells as the invading cell number divided by the total cell number. In several cases, the cells were prepared for scanning electron microscopy following procedures described previously (Toyoizumi and Takeuchi, 1995).

Anti-HNK-1 antibody immunostaining

The cells on the laminin gel after 24 hour culture were fixed with 4% formalin at 4°C for one day, then processed for anti-HNK-1 immunostaining. After washing with Ca^{2+}, Mg^{2+}-free PBS (PBS-), the specimens were preincubated with 5% goat serum for 30-60 minutes at room temperature. Next, 0.5 ml PBS- solution of monoclonal anti-HNK-1 antibody (X60; mouse IgM, Becton Dickinson Co.) supplemented with 0.1% BSA was added to each Petri dish, and incubated for 1.5 hour at 37°C with gentle reciprocal shaking. As a control experiment, 0.5 ml PBS- containing 0.1% BSA without the anti-HNK-1 antibody was used instead of the antibody solution. After washing with PBS- three times, the samples were treated with 2 ml solution of goat anti-mouse IgM colloidal gold (X40; 20 nm in average diameter, EM grade) at 4°C for 2 days. The samples were then washed thoroughly with PBS-. Finally, the localization of the colloidal gold was enhanced with a silver enhancing kit (British BioCell. Co.).

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