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Expression of a high molecular weight cell surface glycoprotein (LETS protein) by preimplantation mouse embryos and teratocarcinoma stem cells

(cell–cell adhesion/embryonal carcinoma cells/inner cell mass/endoderm/embryonic ectoderm)

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ABSTRACT The expression of a high molecular weight cell surface glycoprotein (LETS, fibronectin) by preimplantation mouse embryos as well as cultured teratocarcinoma stem cells was detected by using indirect immunofluorescent staining. When each stage of preimplantation embryonic development was tested for the presence of LETS protein, none was observed on two-cell, four-cell, or eight-cell embryos, or on the morula or the outer cell layer (trophectoderm) of the early or late blastocyst. However, when the inner cell mass was isolated by immunosurgery, positive staining was observed. The intensity of the staining was significantly greater on the inner cell mass isolated from the expanded (day 4) blastocyst than on that from the early (day 3) blastocyst.

Certain established cell lines of teratocarcinoma stem cells (embryonal carcinoma cells) also express cell surface LETS protein. "Nullipotent" (Nulli-SCC-1) as well as pluripotent (PSA 1) embryonal carcinoma cell lines have deposits of LETS protein concentrated in areas of cell–cell contact. In addition, a teratocarcinoma-derived endodermal cell line (PYS) was found to be capable of depositing LETS onto the substratum in a fibrillar network.

Taken together, our results indicate that LETS protein is synthesized at a specific stage of preimplantation mouse embryonic development. In particular, they suggest that LETS protein is a product of the embryonic ectoderm, and that some types of embryonic endoderm are also capable of synthesizing this protein.

A high molecular weight (220,000 dalton) glycoprotein has been shown to be a major cell surface component of many cell types, including fibroblasts (1–7), epithelial cells (8), glial cells (9), myoblasts (10, 11), and endothelial cells (12–14). Originally observed as a large external transformation-sensitive (LETS§) protein present on normal but not on certain transformed cells (for review see ref. 15), the LETS protein has more recently been found to form complexes with collagen (16) and to be a component of the basement membranes in many tissues (17).

Although any direct rôle for the LETS protein in regulating cell proliferation has been ruled out (18–21), current evidence strongly suggests that it can have an important role in mediating cell–substratum adhesion and in maintaining the flattened morphology of cultured fibroblasts (21, 22). In addition, LETS has been implicated as a mediator of certain types of cell–cell interactions. It has been demonstrated to be an agglutinin for sheep erythrocytes (23) and has been proposed to play a role in the protease-induced aggregation of chick embryo fibroblasts (19). Of particular interest is the observation of Chen *et al.* (24) that in sparse cultures of rat fibroblasts the LETS protein is preferentially located in areas of cell–cell contacts. This same distribution is found in normal cultured vascular endothelial cells that grow as a contact-inhibited monolayer but is altered in carcinogen-treated endothelial cells that are capable of overgrowth (12). Taken together, these results suggest that the LETS protein plays a role in the initiation and maintenance of cell–cell contacts.

If LETS protein is important in mediating cell–cell interactions *in vivo*, one likely place to exercise this function would be in the developing embryo. Perhaps nowhere else is it so important to generate and maintain proper cell–cell contact and positioning. Although cell–cell interactions during the later stages of embryonic development have been extensively investigated (for review see refs. 25 and 26), relatively little is known about the nature of the cellular interactions at the earliest (preimplantation) stages of mammalian development.

In this study we have utilized an indirect immunofluorescence assay to investigate the expression of the LETS protein by developing mouse embryos and teratocarcinoma cells. Embryos at each stage of preimplantation development from the two-cell stage through the formation of the expanded blastocyst were tested and, following our observation that LETS protein is expressed only by the inner cell mass (ICM) of the late blastocyst, we assayed cultures of cells derived from teratocarcinomas in order to examine the question of which particular cell type(s) in the embryo might be responsible for the production of LETS protein.

MATERIALS AND METHODS

Embryo and Cell Cultures. All embryos were obtained from random-bred ICR mice (Simenson Laboratories). Embryos were flushed by standard procedures from the oviduct or the uterus one day prior to assay for LETS protein. At that time the zona pellucida was mechanically removed by pipetting and the zona-free embryos were either tested immediately or cultured overnight in a standard embryo culture medium (27) containing 0.3% bovine serum albumin (Sigma). ICMs were isolated from early (day 3) or late (day 4) blastocysts by the immunosurgical procedure described by Solter and Knowles (28), using a rabbit anti-mouse cell serum kindly provided by D. Solter, and guinea pig complement obtained from GIBCO.

Abbreviations (see also § footnote): LETS protein, large external transformation-sensitive protein; ICM, inner cell mass; PYS, parietal yolk sac; CIG, cold-insoluble globulin.

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§ The high molecular weight cell surface glycoprotein discussed in this report is termed LETS protein for convenience and consistency. The reader should note that the following terms have been used to describe identical and closely related proteins: fibronectin, fibroblast surface antigen (FSA), cell surface protein (CSP), galactoprotein A, 250K protein, Z protein, and cold-insoluble globulin (CIG).

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All teratocarcinoma cells were cultured in Dulbecco's modified Eagle's medium (containing 4.5 g of glucose per liter) supplemented with 10% calf serum. The embryonal carcinoma cells used in this study have been described previously (29–31), as has the teratocarcinoma-derived endodermal cell line PYS (parietal yolk sac), kindly provided by John Lehman (32).

Immunofluorescence. Cells cultured on 12-mm glass coverslips were fixed in 3.7% formaldehyde in phosphate-buffered saline or 2% para-formaldehyde and then assayed for the presence of LETS protein with the procedure described by Chen *et al.* (24). Antiserum prepared against human CIG was kindly provided by L. B. Chen. The properties of this monospecific antiserum have been described by Chen *et al.* (24) and by Burridge (33). Intact embryos, ICMs, and embryonal carcinoma cell clumps cultured in suspension were stained prior to fixation by transfer to a 50-μl drop of antiserum diluted (1:80) in phosphate-buffered saline. After incubation at 37° for 30 min, they were washed three times by sequential transfer to medium containing 0.3% bovine serum albumin. They were subsequently incubated for 30 min at 37° in a 50-μl drop of goat anti-rabbit immunoglobulin conjugated to fluorescein (Meloy), washed an additional three times, and then fixed. Individual embryos, ICMs, or embryonal carcinoma clumps were then transferred to a 50-μl drop of water on a glass microscope slide and gently trapped between the slide and a coverslip that was resting on a small drop of Lubriseal grease at each of its corners. Fluorescence was observed by using a Leitz Orthoplan microscope under epi-illumination and photographed with Kodak Tri-X film in a Leitz Orthomat camera. In all cases in which positive fluorescence was observed, the specificity of the staining was determined by control experiments in which nonimmune rabbit serum was used in place of the anti-CIG antiserum or by experiments in which fluorescence was blocked by adding excess purified human CIG to the anti-CIG antiserum.

RESULTS

The results of immunofluorescence tests for the expression of LETS protein by mouse embryos at various stages of preimplantation development are shown in Fig. 1. Cleavage stage embryos (two-cell, four-cell, eight-cell) showed no detectable surface LETS protein. Negative results were also obtained with the early and late morula (approximately 16–32 cells). At the blastocyst stage (approximately 64 cells) the embryo contains two distinct cell types: an outer layer of trophectoderm that encloses a fluid-filled cavity, the blastocoel, and the pluripotent ICM at one end of the blastocoel. In order to determine whether LETS protein is expressed by either of these cell types, two separate experiments were performed. First, we assayed intact blastocysts, thereby testing for the presence of LETS protein on the surface of the outer, trophectodermal cells. Second, we assayed for the presence of LETS protein on ICMs, which had been isolated from intact embryos by immunosurgery. Whereas the trophectoderm was found to be completely negative, the ICM isolated from the early (day 3) blastocyst showed slight reactivity with the anti-LETS antibody (Figs. 1 and 2). When these tests were repeated using day 4 blastocysts, the trophectoderm was still completely negative but the ICMs isolated from these blastocysts had become strikingly positive. The LETS protein can be seen as a matrix in the intercellular spaces as well as in the form of large fibrils that crisscross the whole surface of the ICM (Fig. 2).

At the time at which LETS protein is first detectable, the ICM has begun its differentiation into two cell types: those cells on the surface of the ICM that is exposed to the blastocoel are forming primitive endoderm, while the remaining ICM cells are forming embryonic ectoderm. In order to determine which of these two cell types is responsible for the appearance of the LETS protein, several experiments were performed. In the first,

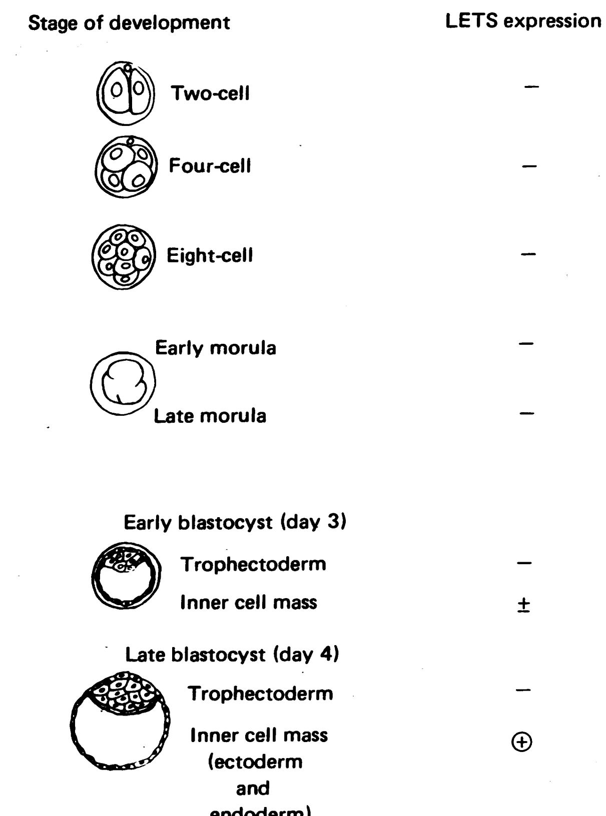

FIG. 1. Expression of LETS protein on preimplantation mouse embryos.

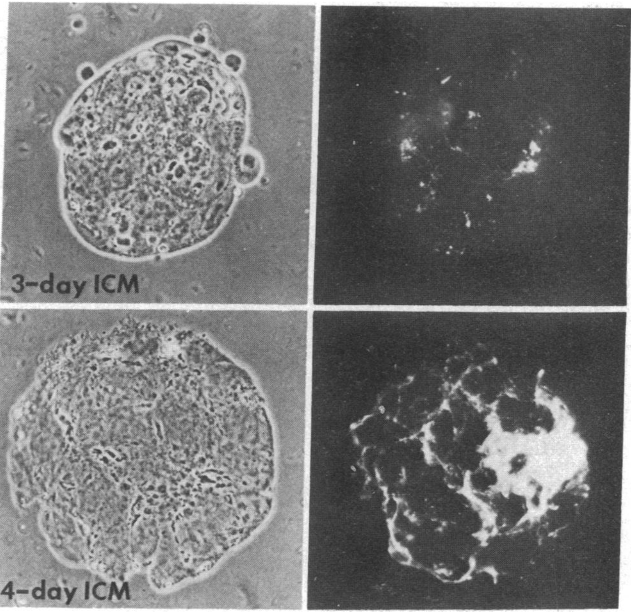

FIG. 2. Distribution of LETS protein on isolated ICMs. ICMs were isolated from early (day 3) or late (day 4) blastocysts and assayed by indirect immunofluorescent staining. (*Left*) Phase contrast photomicroscopy; (*Right*) fluorescent photomicroscopy of same field. Bright fluorescence is observed only on the day 4 ICM. (×590.)

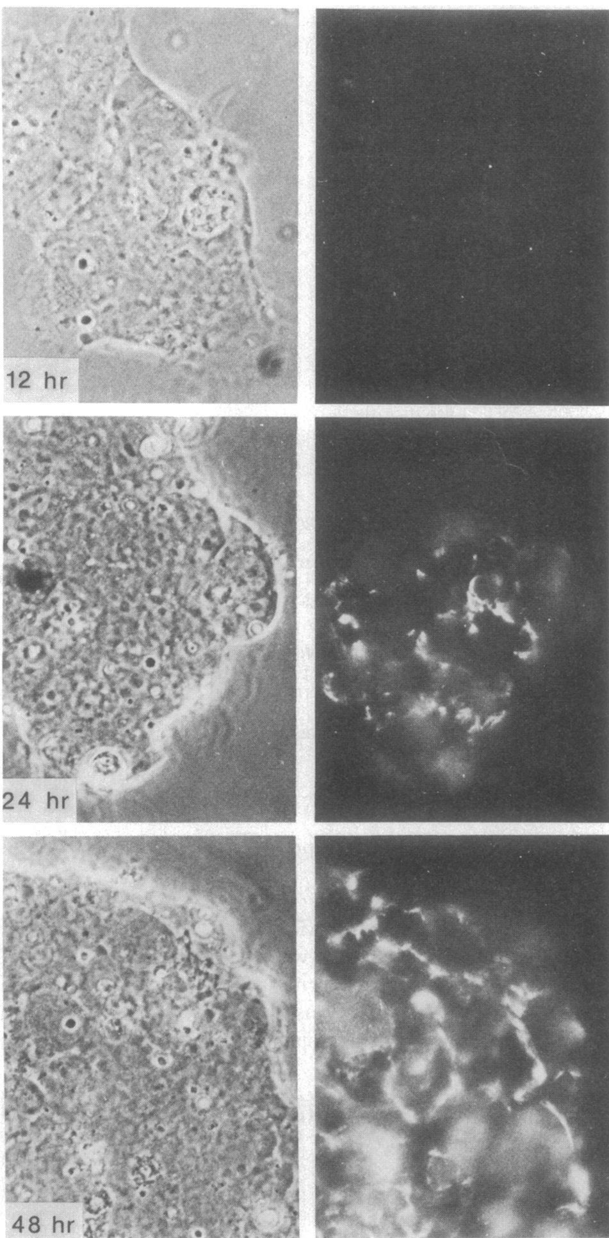

FIG. 3. Production of LETS protein by teratocarcinoma stem cells (embryonal carcinoma cells). Photography as in Fig. 2. Cultures of Nulli-SCC-1, a nullipotent embryonal carcinoma cell line, were treated with 0.25% trypsin to detach the cells from the tissue culture dish and to remove LETS protein from the cell surfaces. Twelve hours after replating, no LETS protein is detectable by immunofluorescence. After 24 hr small amounts of LETS protein are visible on the cell surfaces, and by 48 hr significant amounts are apparent in the intercellular spaces. (×570.)

ICMs were isolated from late blastocysts and cultured for two days, after which time a complete outer layer of endoderm has formed. Immunofluorescence tests indicated that the endodermal cells did not express LETS protein on their outer surfaces.

A second approach to the problem of which embryonic cells express LETS is through the use of teratocarcinoma cells. Several of these tumor-derived cell types are similar to the cells of the early embryo. In particular, embryonal carcinoma cells, which are the stem cells of teratocarcinomas, are known to be closely similar to the pluripotent cells of the normal embryo (for review, see refs. 34 and 35) and in particular to the embryonic ectoderm (36). To determine whether cultures of a pluripotent embryonal carcinoma cell line (PSA 1) synthesize LETS protein, the cells were plated at high density in the absence of feeder cells. Under these conditions, the cells initially form undifferentiated flattened aggregates which are attached to the tissue culture substratum (29–31). Immunofluoresence assays of these attached aggregates indicated that LETS protein is found on the surface of these cells and is frequently concentrated in the areas of intercellular contacts.

In order to make certain that the LETS that we detected in these undifferentiated pluripotent cultures was a consequence of neither incipient differentiation nor prior coculture of the embryonal carcinoma cells with fibroblastic feeder cells, we also tested a "nullipotent" embryonal carcinoma cell line, Nulli-SCC-1. Although these cells are morphologically and biochemically similar to the pluripotent ones (29, 36), they are apparently incapable of differentiation both *in vivo* and *in vitro* and also need never be cocultured with fibroblastic feeder cells (29, 30). Our immunofluororescence assays indicated that these cells also deposit LETS protein on their cell surfaces. Fig. 3 shows that when these cells are removed from the culture dish with 0.25% trypsin and replated, no LETS protein is seen for at least the first 12 hr of culture. LETS protein becomes detectable within 24 hr and is deposited in larger amounts by 48 hr of culture. These results indicate that the LETS protein detectable in the pluripotent cultures was synthesized by the undifferentiated embryonal carcinoma cells. Because these cells have been shown to be most similar to the embryonic ectoderm (36), the finding that undifferentiated teratocarcinoma stem cells express LETS protein on their surfaces suggests that at least part of the LETS protein observed on ICMs could be produced by the embryonic ectoderm.

To make further use of the teratocarcinoma cells as a model for studying the expression of LETS protein in the early embryo, we cultured the pluripotent PSA 1 cells under conditions that are conducive to their differentiation (29–31). As with the ICMs, the first stage of their differentiation is the formation of a layer of endoderm over the whole outer surface of embryonal carcinoma cell clumps. When these two-layered "embryoid bodies" were assayed for LETS expression we found that, as with the cultured ICMs, there was no detectable LETS protein on the outer surface of the endodermal cells. Interestingly, however, there was some indication that the teratocarcinoma-derived endoderm might be capable of synthesizing LETS protein. In some instances we observed bright fluorescence on the extracellular material that lies between the outer endodermal cell layer and the inner embryonal carcinoma core. To test whether this LETS-positive material is produced by endodermal cells, we cultured an endodermal cell line, PYS, derived from the endoderm of teratocarcinoma embryoid bodies (32). Fig. 4 demonstrates that these PYS cells do indeed produce considerable quantities of LETS protein, and that the protein is distributed in the form of long fibrils that can be seen running between the cells. This pattern is similar to that observed with cells such as fibroblasts and endothelial cells that secrete basement membrane materials (12). It should be noted, however, that the LETS protein production by PYS cells was not the same under all culture conditions. For example, we have observed that the PYS cells deposit much greater quantities of LETS protein when grown in the presence of calf serum then they do when grown with fetal calf serum. This result is consistent with the finding of Chen *et al.* (37) that the production of LETS protein can be influenced by nutritional conditions such as the presence of certain growth factors. However, growth in different types of serum did not affect LETS protein pro-

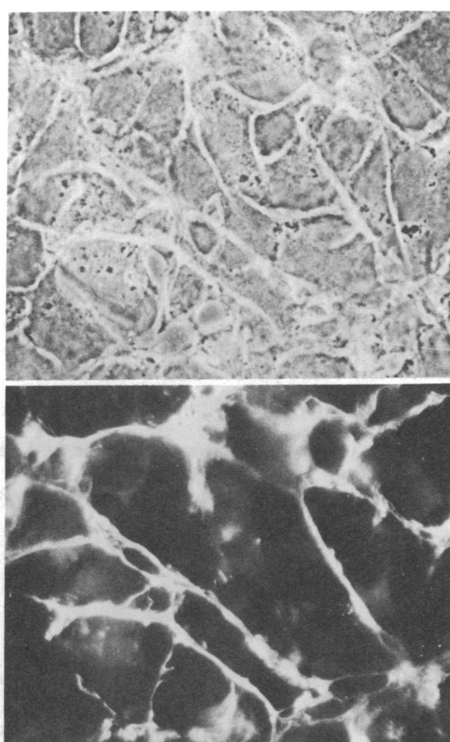

FIG. 4. Production of LETS protein by a teratocarcinoma-derived endodermal cell line (PYS) cultured in media containing 10% calf serum. (*Upper*) Phase contrast with simultaneous fluorescent photomicroscopy; (*Lower*) fluorescent photomicroscopy alone. (× 400.) The LETS protein is found not only in the intercellular spaces but also in long extracellular fibers that cross the surface of several cells.

duction by any of the other cell types described in this report.

DISCUSSION

The results described above indicate that during preimplantation development of the mouse embryo, expression of the LETS protein is correlated with a specific differentiative event. Thus, when the inner cell mass from the early blastocyst is assayed for the expression of cell surface LETS protein, little if any is detectable. However, 24 hr later, when the differentiation of the ICM cells to endoderm and ectoderm has begun, considerable surface deposits of the LETS protein are observed. In contrast, the outer trophectodermal cell layer, which is the third cell type present in the embryo at this late blastocyst stage of development, shows no detectable LETS protein. The trophectodermal cells are equally negative at earlier stages of development, as are the cells of the embryo prior to the differentiation of trophectoderm and inner cell mass (cleavage stage embryos).

Our results with embryos do not indicate which of the cell types present in the ICM is responsible for synthesis of the LETS protein. However, our experiments with teratocarcinoma stem cells may help to resolve this question. Recent experiments comparing the patterns of protein synthesis in the embryonal carcinoma cells described above and in mouse embryos at various stages of development have indicated that the teratocarcinoma stem cells are closely similar to the embryonic ectoderm (36). Thus our finding that teratocarcinoma stem cells express the LETS protein is consistent with the idea that the embryonic ectoderm is capable of synthesizing this protein. Expression of the LETS protein does not appear to be related to the differentiative capacity of the cells, because it can be detected on nullipotent as well as pluripotent embryonal carcinoma cells. However, not all embryonal carcinoma cell lines synthesize the LETS protein. Immunofluorescence assays of the F9 and PCC4/A/1 embryonal carcinoma cells (38) were completely negative (data not shown). It is of interest that the LETS-negative embryonal carcinoma cells have considerably less tendency to form tightly packed aggregates than do the LETS-positive embryonal carcinoma cell lines. This is consistent with previous reports of an involvement of LETS protein in cell–cell adhesiveness (19, 23). Our results are also consistent with the observations of Wartiovaara *et al.* (39) that not all embryonal carcinoma cell lines express this protein.

It should also be noted that the quantity of LETS protein produced by the embryonal carcinoma cells appears to be significantly less than that produced by cells such as fibroblasts or endothelial cells that secrete this protein as a basement membrane component. The intercellular LETS deposits seen on the embryonal carcinoma cells can only be detected by use of the highly sensitive indirect immunofluorescent staining technique and are not observed when these cells are iodinated with ^{125}I and assayed using slab gel electrophoresis and autoradiography (R. O. Hynes and G. R. Martin, unpublished data).

The question of whether or not the primitive embryonic endoderm can synthesize the LETS protein remains unanswered. The positive results we obtained with teratocarcinoma-derived endodermal cells (PYS) do not directly address this question, because these cells represent a different type of endoderm (parietal endoderm) than that present on the blastocoelic surface of the ICM (visceral endoderm). Thus, while the finding that PYS cells produce a fibrillar matrix of LETS protein does not indicate whether or not this protein is produced by the embryonic endoderm of the late blastocyst, it may be indicative of LETS production by the parietal endoderm of the postimplantation embryo.

The work described here with preimplantation embryos raises the question of whether the LETS protein continues to be expressed after implantation. Using indirect immunofluorescence assays of paraffin-embedded embryos, Wartiovaara *et al.* (40) have been able to detect LETS protein on some basement membranes but not on the cell surfaces of postimplantation embryos. This suggests that the LETS protein expression we have detected on the surface of the ICM immediately prior to implantation is transitory and that further differentiation involves negative as well as positive controls on LETS protein expression. Our results therefore imply that LETS protein expression might play an important, stage-specific role in the normal peri-implantation development of the embryo.

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