Le^X Structure Enhances Myocardial Differentiation from Embryonic Stem Cells

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ABSTRACT. α -1,3-Fucosyltransferase (Fuc T IV) cDNA was placed under the control of β -actin, cytomegalovirus enhancer/promoter and transfected into embryonic stem cells. The transfected cell clones with integrated cDNA (positive clones) differentiated more efficiently into myocardial cells than the clones without integrated cDNA (negative clones) or parental cells. Furthermore, myocardial cells differentiated from the positive clones survived longer than those differentiated from the negative clones or parental cells. These results indicate that Lewis X structure, the product of Fuc T IV, enhances myocardial differentiation. The mechanism of the phenomenon is discussed in relation to integrin action.

Cell surface carbohydrates change dramatically during differentiation and development (10, 11). For example, Lewis X (Le^X) structure, Gal β 1 $\rightarrow$ 4 (Fuc α 1 $\rightarrow$ 3) GlcNAc epitope, comes to be expressed in late 8 cell stage during mouse embryogenesis, and after implantation is strongly expressed in embryonic ectoderm, a part of visceral endoderm and trophoblast (6). As the embryogenesis proceeds, its expression becomes progressively restricted, and on the 14th day of gestation, it is detectable only in highly specific cells such as primordial germ cells (6, 18). The biological meaning of the developmentally restricted expression is only partly understood. Le^X structure is apparently involved in compaction, which is an increased cell adhesion phenomenon in early preimplantation embryos (1, 5). Le^X structure has been shown to be self complementary and to promote intercellular adhesion (4). On the other hand, we proposed that Le^X structure enhances integrin-mediated cell-substratum adhesion, based on immunological inhibition (13) and cDNA transfection of α 1 $\rightarrow$ 3 fucosyltransferase (Fuc T IV) (16). However, the possible effects of Le^X structure on cell differentiation have not been examined. In order to address the question, we transfected Fuc T IV cDNA into embryonic stem cells, which differentiate into many cell types *in vitro*, and examined whether the direction of differentiation is altered in the transfected cells.

MATERIALS AND METHODS

Culture and differentiation of embryonic stem cells. D3 embryonic stem cells (3) were kindly provided by Dr. Rolf Kemler, and were cultured in Dulbecco-modified minimum essential medium (DMEM) containing 15% fetal calf serum and 10³ units/ml leukemia inhibitory factor (LIF), using the feeder layer of mitomycin C-treated SL10 cells (8). For induction of differentiation, the cells detached from the feeder layers (1 $\times$ 10⁶) were cultured in DMEM with 15% fetal calf serum on a Falcon 1,005 dish. Cells formed aggregates and differentiated into embryoid bodies. After 7 days of culture, cystic embryoid bodies formed were picked up and 10 of them were transferred to slide chamber (Nunc, 177380) and cultured in the same medium. Embryoid bodies were attached to the surface of a culture dish, and differentiated cells became evident in the cell layer. The area of myocardial cells was identified as beating muscle cells by observation using Olympus inverted phase contrast microscope model IMT-2. To quantify the degree of myocardial differentiation, the total area of the cell sheet was recorded by photomicrograph, and the area of myocardial cells were estimated by video recording of cell movements.

Transfection. cDNA of human α -1,3-fucosyltransferase (Fuc T IV) (9) was kindly given by Dr. J.B. Lowe and was inserted into PCAGGS, which has β -actin and cytomegalovirus enhancer/promoter and has neomycin resistance gene (12), as described previously (16). The fusion gene was introduced into D3 embryonic stem cells by electroporation using Gene Pulser (Bio Rad) at 500 v/cm, capacitance 960 μ FD and time constant, 14.5. Cells surviving after treatment with 400 μ g/ml G418 were cloned.

Staining with a monoclonal antibody against cardiac myo-

Abbreviations: Fuc T IV, α -1,3-Fucosyltransferase; Le^X, Lewis X; PBS, Dulbecco's phosphate buffered saline; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl-15 mM sodium citrate.

sin heavy chain. Cells grown on chamber slide (Nunc, 2 well 177380) were washed twice with Dulbecco's phosphate-buffered saline [PBS(+)], soaked in methanol at -20°C for 10 min and in acetone at -20°C for 1 min, and washed with PBS(+) twice. The cells were then soaked in 0.01% NaBH_4 in PBS(+) and in normal goat serum diluted 20-fold with PBS(+), and washed with PBS(+) twice. Specimens were incubated with monoclonal mouse IgG antibody against bovine ventricular myosin heavy chain α/β (Chemicon International Inc.) diluted 10-fold with 1% bovine serum albumin in PBS(+) at 4°C overnight, and washed with PBS(+) three times. They were then incubated with affinity-purified goat anti mouse IgG labeled with fluorescein isothiocyanate (Jackson Immunoresearch Lab.) diluted 20-fold with PBS(+) for 1 h at room temperature, and washed with PBS(+) five times. Specimens were observed using an Olympus fluorescence microscope BX-60. In the control run, the first antibody was replaced with 1% bovine serum albumin in PBS(+).

Fig. 1. Southern blot analysis to classify the transfected embryonic stem cell clones into those with the Fuc T IV cDNA and those without it. DNA isolated from cell clones was digested with *Bam* HI and was analyzed by agarose gel electrophoresis as described previously (16). After transfer to nitrocellulose membrane, the membrane was probed with [^{32}P]-labeled *Pvu* II fragment of Fuc T IV cDNA. Washing conditions were as follows: $2\times\text{SSC}$, 0.1% SDS, 55°C , 5 min twice and $0.1\times\text{SSC}$, 0.1% SDS, 55°C , 30 min. Clones 10 and 4 were judged to be positive, while clones 9, 6, and 3 were classified to be negative. An arrow indicates the position where the Fuc T IV *Bam* HI fragment migrated.

Other Methods. RNA was prepared by the guanidium isothiocyanate method (2). Southern and Northern blot analysis was performed as described before (16). *Pvu* II/*Pvu* II fragment of Fuc T IV cDNA (nucleotide number 202–1257) labeled with [^{32}P] using Megaprime DNA labeling system (Amersham) was used as a probe. Immunohistochemical detection of Le^x antigen was performed as described (16) using SSEA-1 monoclonal antibody (15) as the first antibody.

RESULTS

Human α -1,3-fucosyltransferase (Fuc T IV) cDNA under the control of β -actin and cytomegalovirus enhancer/promoter was transfected into D3 embryonic

Fig. 2. Northern blot analysis to verify that the positive clones expressed Fuc T IV cDNA. Ten μg of total RNA was separated by electrophoresis in 1% agarose. After transfer to nitrocellulose membrane, it was probed with [^{32}P]-labeled *Pvu* II fragment of Fuc T IV cDNA. The washing conditions are the same as those used in Fig. 1. An arrow indicates the position where Fuc T IV mRNA migrated. Both clones 2 and 4 expressed Fuc T IV cDNA.

stem cells. Several G418 resistant clones were picked up and analyzed by Southern and Northern blot, and were classified into those with integrated Fuc T IV cDNA (positive clones) and those without it (negative clones)

Fig. 3. Myocardial differentiation from D3 embryonic stem cells. A, Unfixed cells observed by phase contrast microscopy: the area of the cell sheet marked by a dashed line was considered to be myocardial cells by rhythmical cell movement. B and C, fixed cells observed by Normaski differential-interference-contrast microscopy (B) or by fluorescence microscopy (C) after staining with anti-cardiac myosin. Bar: 100 μ m

(Fig. 1 and 2).

The differentiation capabilities of the positive clones, negative clones and the parental embryonic stem cells were comparatively analyzed. We found that the positive clones were enhanced in myocardial differentiation. The degree of myocardial differentiation was evaluated from the area of beating cells (Fig. 3A), which were identified as myocardial cells by specific staining with anti-myocardial myosin (Fig. 3C): the beating muscle cells were intensely stained, but scarcely any staining was observed in the control staining. The area of myocardial differentiation microscopically determined was significantly larger in cell sheets derived from the positive clones than those derived from the negative clones or those from parental cells (Table I). Furthermore, myocardial cells differentiated from the positive clones sur-

Fig. 4. Expression of Le^x antigen in cells differentiated from Fuc T IV positive cells. The area of myocardial cell differentiation (A) and that of chondrocytes (B) showed strong immunofluorescence which indicates the presence of Le^x antigen reacting with SSEA-1 monoclonal antibody. Bar: 100 μ m

Table I. EFFECTS OF TRANSFECTION WITH FUC T IV ON MYOCARDIAL DIFFERENTIATION FROM ES CELLS.

Days	Area of myocardial Differentiation (per cent of total cell area)					
	0~4	5~7	8~10	11~13	14~16	17~19
D3 wild type cell clones (n=4)	0	3.2±0.6	5.8±1.9	4.2±1.2	0.9±1.8	0
D3 cells transfected with α -1,3-fucosyltransferase cDNA						
Le ^x -positive clones (n=4)	0	14.0±5.0	18.8±6.8	20.8±7.6	18.2±6.8	12.0±2.7
Le ^x -negative clones (n=4)	0	5.3±1.2	5.9±1.2	5.4±0.8	a	a

For each clone, two embryoid bodies were observed, and the average value of myocardial differentiation was statistically analyzed.

*: $P < 0.05$ **: $P < 0.001$ a: observation was not performed

vived longer. In the cell layer from the positive clones, about 12% of the cells were beating muscle cells at 17–19 days after induction of differentiation. On the other hand, scarcely any beating muscle cells were observed in cell layer differentiated from parental D3 cells on 17–19 days after transfer to chamber slides (Table I).

We stained the differentiated cell layer by anti-SSEA-1, which reacts with Le^x structure, namely Gal $\beta 1 \rightarrow 4$ (Fuc $\alpha 1 \rightarrow 3$) GlcNAc, to examine whether transfected α -1,3-fucosyltransferase cDNA really alters cell surface structure. In cells cultured in chamber slides for 10 days, intense staining was found only in beating muscle cells (Fig. 4A) and aggregated cells with the morphology of chondrocytes (Fig. 4B), which were identified by Alcian blue stain. In cell layers differentiated from parental D3 cells, only diffuse staining was observed in the myocardial cells (data not shown). No enhancement of chondrocyte differentiation was observed by transfection with α -1,3-fucosyltransferase cDNA.

DISCUSSION

We found that transfection with α -1,3-fucosyltransferase cDNA enhances myocardial differentiation and survival of the differentiated myocardial cells *in vitro*. Northern blotting and staining with anti-SSEA-1 confirmed that the transfected cells with integrated Fuc T IV cDNA actually express the cDNA and form the glycosylated product. Since embryonic stem cells express SSEA-1, the effects of fucosyltransferase transfection cannot be observed at the level of embryonic stem cells. After differentiation, most cells lose strong expression of SSEA-1. We observed that only the myocardial cells and the cells identified to be chondrocytes strongly express SSEA-1 among the many types of differentiated cells. The availability of acceptor structures for the fucosyltransferase action are probably limited to these cells. A similar observation was made in transgenic mice with α -1,3-galactosyltransferase and those with α -1, 3/4 fucosyltransferase (Fuc T III) (7, 14). In any event, increased expression of SSEA-1 in myocardial cells indicates that the cell surface carbohydrate profile is actually changed as a result of transfection.

The enhanced myocardial differentiation and survival

of the differentiated cells were repeatedly observed in different Fuc T IV (+) clones, and thus in some way should be related to the altered cell-surface structure. Enhanced myocardial differentiation can be caused by enhanced Le^x expression in precursor cells leading to myocardial cells, although because of inability to identify the precursor cells, direct demonstration of increased Le^x expression in the putative precursor cells could not be performed. We previously showed that transfection with α -1,3-fucosyltransferase led to increased integrin-mediated cell-substratum adhesion (16). Furthermore, integrin action is required for cardiac development, since gene targeting of integrin $\alpha 4$ subunit resulted in impaired heart formation (17). It is possible that increased integrin action is the cause of increased myocardial differentiation upon transfection with α -1,3-fucosyltransferase cDNA. The persistence of differentiated myocardial cells after 17–19 days in chamber slide culture is most probably due to enhanced survival of differentiated cells, although the possibility should not be excluded that increased survival of precursor cells results in persistence of the differentiated cells.

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