Expression of αGal Epitope on COS-7 Cells Transfected with Bovine α1-3 Galactosyltransferase

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The polysaccharide antigen, called αGal epitope is ubiquitously found in all animals except for humans and old world monkeys. An αGal epitope is a main xenoantigen which hampers the success of the clinical xenotransplantation. The αGal epitope is synthesized by an enzyme, α1-3 galactosyltransferase (α1-3GT). Thus, disruption of the α1-3GT gene in large animals is one strategy for the successful xenotransplantation. In the present study, we isolated α1-3GT cDNA of Japanese Black Cattle (JBC), and transfected it into an α1-3GT negative COS-7 cell line to demonstrate that the cDNA was functional. The α1-3GT cDNA of JBC was amplified by a polymerase chain reaction. The α1-3GT cDNA was cloned to an expression vectors, named N3GA and transfected into α1-3GT negative COS-7 cells. Cell surface expression of αGal epitope was examined by immunofluorescent staining and flow cytometry with IB4 lectin. Transfected COS-7 cells were positive for the immunoreactive IB4 lectin examined at 24 hours after the transfection. Then, transfected COS-7 cells were selected with neomycin for 10 days. Flow cytometry of COS-7 cells performed 4 weeks after the transfection demonstrated positive log shift for αGal epitope. In conclusion, we isolated α1-3GT cDNA of JBC and obtained stable cell line expressing the gene.

Introduction

Xenotransplantation is hampered by hyperacute rejection (HAR). HAR begins with the binding of the natural antibodies in human serum to αGal epitope expressed on the endothelia of xenogeneic donors. It is known that αGal epitope is synthesized by an enzyme, α1-3 galactosyltransferase (α1-3GT). The functional α1-3GT is ubiquitously found in animals except for humans and old world monkeys

Strategies have been investigated to overcome HAR. Those strategies include elimination of natural antibodies from the recipient, production of transgenic pigs expressing human complement regulating factors and inhibition of αGal expression by introducing enzymes which compete with α1-3GT

The most rational approach to overcome HAR, which is targeting α1-3GT gene, has been successful only in murine model by ES cell technology. However, recent advance in reproductive technology emerged the possibility to produce

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α1-3GT knock-out cattle. It has been suggested that the nucleus of the somatic cells, in which the particular gene is disrupted, can be transferred to enucleated oocytes. Then, the genetically engineered animals can be generated.

The isolation of the functional bovine α1-3GT cDNA is the essential process in the construction of the targeting vector if bovine is used as the source of xenotransplant. In the present study, we isolated α1-3GT cDNA from Japanese Black Cattle (JBC), and demonstrated that functional expression of the gene by introducing cDNA into an α1-3GT negative COS-7 cell line.

Materials and Methods

1. Construction of bovine α1-3GT cDNA expression vector

The present study was performed with the permission of Tokyo Women's Medical University, Institute of Laboratory Animals (#99-142).

Peripheral blood of Japanese Black Cattle (JBC) was obtained from ZEN-NOH (Hokkaido, Japan). Total RNA was isolated by ISOGEN (NIPPON GENE, Tokyo, Japan). The oligonucleotides for polymerase chain reaction (PCR) primers for bovine α1-3GT (GA-S1: 5'-AGCTCAGTAGAAC-TTGTACTTTT-3', GA-AS3: 5'-TTACTTGGACACATTAGTCTAC-3') were synthesized by IWAKI Co. Ltd. (Chiba, Japan), based on the data from GenBank (ACC# J04989). An 1358 bp DNA fragment was amplified by the RT-PCR (SUPERSCRIPT™, Preamplification System, GIBCO, Rockville, MD), using GA-S1 and GA-AS3 as primers (94 °C-1 min, 55 °C-1 min, 62 °C-1 min, 20 cycles). This fragment encompassed the whole coding sequence of bovine α1-3GT cDNA including the translation start site and a stop codon. The fragment was subcloned to pGEM®-T Vector (Promega, Madison, WI). The vector was cut at the Apal and SalI sites, and ligated to pBluescript KS (-). Then the fragment was cut at the Apal and BamHI sites and cloned to pEGFP-N 3 (CLONTECH, Palo Alto, CA) to construct N3GA.

2. Cells and culture conditions

COS-7 cells (COS (-)) and COS-7 cells transfected with bovine α1-3GT cDNA (COS (+)) were cultured in a 10 cm tissue culture plate in 10 ml of Dulbecco's modified eagle medium (DMEM, GIBCO) containing 10% fetal calf serum (FCS) in 5% CO₂ incubator at 37 °C. Cells were collected and passaged at the time of the confluency.

3. Transfection

To introduce N3GA into α1-3GT negative COS-7 cell line, we used LIPOFECTAMINE™ (GIBCO). Briefly, 1 × 10⁶ COS-7 cells were plated in a 24 well tissue culture plate. COS-7 cells were incubated for 48 hours in 5% CO₂ incubator at 37 °C. Three hundred microliters of OPTI-MEM® (GIBCO) containing 10 µg of LIPOFECTAMINE™ was incubated at room temperature for 30 min. Then 3.2 µg of N3GA was added into the OPTI-MEM®. After 15 min of incubation at room temperature, OPTI-MEM® containing N3GA was transferred to COS-7. After 5 hours incubation in 5% CO₂ incubator at 37 °C, OPTI-MEM® was replaced with 1 ml of DMEM containing 10%FCS. COS-7 cells were incubated with 5% CO₂ incubator at 37 °C for 24 hours.

4. Selection of COS(+)

Twenty-four hours after the transfection, cells were resuspended in 1 ml of DMEM. Ten microliters of cell suspension was transferred to 24 well tissue culture flasks in 1 ml of DMEM with 10% FCS, containing G418 (GENETICIN, GIBCO) at 1,000 µg/ml. Culture medium was replaced every 3 days. After 10 days of culture, 1 × 10⁷ cells were transferred to 10 cm tissue culture plate without G418. Colonies were collected with a cloning ring (IWAKI). Cells were cultured and analyzed using a fluorescent microscope and flow cytometry.

5. Immunofluorescent staining

Twenty-four hours after the transfection, cells were stained in a 4 well chamber slide (IWAKI,
Chiba) in 100 μl of PBS containing 20 μg/ml FITC conjugated BS-I ISOLECTIN B4 (IB4 lectin, Sigma, St. Louis, MO). IB4 lectin recognizes terminal galactosyl epitope in the α linkage. After 1 hour of incubation at room temperature, cells were examined by the fluorescent microscope.

6. Flow cytometry

Staining of COS(−) and COS(+) was carried out as follows. Cells were collected from tissue culture plates by scraping without trypsin or EDTA. Approximately 5 x 10^6 cells were stained in 100 μl of PBS containing FITC conjugated IB4 lectin for 30 min at 4 °C. Cells were washed twice with PBS and analyzed by flow cytometry on a FACscan (Becton Dickinson, Mountain View, CA).

Results

1. Construct of N3GA

Figure 1 shows the construct of expression vector N3GA. N3GA contained a 1.358 kb fragment, coding functional domain of bovine α1-3GT cDNA, following CMV promoter. The sequence of bovine α1-3GT cDNA included the translation start site and a stop codon. N3GA also contained a neomycin resistant gene for the selection of positive clones.

2. Immunofluorescent staining

To examine whether transfected N3GA cDNA produce functional bovine α1-3GT to express αGal on cell surface, we stained COS(−) and COS (+) with IB4 lectin which recognizes the terminal galactose epitope in the α linkage. As shown in Fig. 2, at 24 hours after the transfection, COS (+) were positive for the αGal epitope (Figs. 2 A and a), whereas COS(−) were negative (Figs. 2 C and c). The immunofluorescent staining of COS(+) without IB4 lectin was negative (Figs. 2 B and b). These findings indicate that αGal expression detected by the immunofluorescent staining were due to α1-3GT cDNA, but not to GFP in N3GA.

3. Flow cytometry

To obtain stable cell lines which carry αGal surface expression, COS(+) were selected with neomycin for 2 weeks.

As shown in Fig. 3, COS(+), but not COS(−) showed positive log shift stained with IB4 lectin, 4 weeks after the transfection. This finding indicates that the cloned cDNA carried a functional α1-3GT.

Discussion

Current progress in transplantation biology

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![Fig. 1 Construct of N3GA](image)

ATG and TGA are the translation start site and a stop codon in the N3GA, respectively.
Fig. 2  IB4 lectin staining

A, B and C are images of immunofluorescent staining. a, b and c are images of bright field view.
COS-7 (+) were stained with IB4 lectin (A, a) or without IB4 lectin (B, b). COS-7 (−) were stained with IB4 lectin (C, c).
has shown αGal epitope as a major xenoreactive leading to HAR in xenotransplantation. The
αGal epitope is generated by α1-3GT and is observed in animals except for old world monkeys
and humans. Genes encoding α1-3GT in old world monkeys and humans had mutated and became pseudogene. The most rational approach to overcome HAR may be a generation of α1-3GT knock-out xenogeneic donor. Cloning of α1-3GT cDNA in a certain species is mandatory to construct the knock-out.

Although pigs have been suggested as potential xenogeneic donors, current technologies in cloning could be easier in sheep and cattle than pigs. This prompted us to consider cattle as alternative xenogeneic donors.

Although amino acid sequences of α1-3GT cDNA have already been reported in some animals including cattle, it was necessary to obtain α1-3GT cDNA of JBC for the process of producing α1-3GT knock-out cattle utilizing cloning.

In this study, α1-3GT cDNA was cloned from the same JBC used for the cloning. The amino acid sequence of the JBC α1-3GT cDNA was not different from that previously reported except for a few amino acids. It has been shown that α1-3GT shares amino acid sequences between animal species, especially in the sequence of the catalytic domain.

N3GA was established by flanking JBC α1-3GT cDNA into an expression vector which contained green fluorescent protein (GFP). GFP was detected by a fluorescent microscope and one channel of the flow cytometer. It is unlikely that positive αGal expression on COS(+) was the result of GFP, because the GFP mRNA can not be translated due to the presence of the stop codon in the construct of N3GA (Fig. 1).

Immunofluorescent staining and flow cytometry of COS(+) demonstrated that N3GA carried the functional domain of JBC α1-3GT cDNA. A relatively low positive log shift was detected in flow cytometry compared with immunofluorescent staining. This discrepancy may be attributed to the incubation time after the transfection. We selected COS-7 cells with neomycin for 2 weeks after the transfection. During this period, some COS-7 cells lost transfected genes. N3GA was also transfected into other cell lines, Vero and Hela (data not shown). Vero showed the equivalent level and Hela showed lower levels of αGal expression compared with COS-7 cells.

Based on the data of the current study, produc-
tion of α1-3GT knock-out cattle is underway. We isolated genomic sequences of JBC α1-3GT and constructed targeting vector. Fetal fibroblast cell lines of JBC were established and used as a donor cell for nuclear transfer.

In conclusion, we amplified α1-3GT cDNA of JBC and made an expression vector, N3GA, containing α1-3GT cDNA. The transfection experiment of COS-7 cells with N3GA demonstrated that N3GA carried the functional domain of JBC α1-3GT.

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References

ウシ α1-3 galactosyltransferase の COS-7 細胞への導入

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αGal 抗原は異種抗原として知られ、ヒトおよび旧世界サル以外の生物に広汎に存在し、異種移植における超急性拒絶反応の原因抗原と考えられている。この αGal 抗原は α1-3 galactosyltransferase (α1-3GT) によって合成される。異種移植の分野では、α1-3GT を様々な方法で修飾することにより、移植可能な動物の作製が試みられている。本実験では、黒毛和牛から α1-3GT を単離し、これを αGal 陰性の COS-7 細胞に導入し、我々のクローニングしたウシ α1-3GT が機能するかどうかについて検討した。

黒毛和牛末梢血から、RT-PCR 法を用いて α1-3GT cDNA をクローニングした。これを、ネオマイシン耐性遺伝子を含むベクターに挿入し、αGal 抗原発現ベクターを作製した (N3GA)。N3GA をリポフェクション法により、αGal 抗原陰性である COS-7 細胞に導入した。N3GA 導入翌日に IB4 レクチンを用いた蛍光抗体染色を行った。N3GA を導入した COS-7 細胞 (COS (+)) では、αGal 抗原陽性所見を認めた。また、N3GA 導入 2 週間後に、同じく IB4 レクチンを用いて行った flow cytometry でも COS (+) では αGal 抗原陽性所見を認めた。

以上より、我々が黒毛和牛からクローニングした α1-3GT は酵素機能を有しており、異種細胞においても機能することが示唆された。