Establishment of an embryonic stem (ES) cell line derived from a non-obese diabetic (NOD) mouse: in vivo differentiation into lymphocytes and potential for germ line transmission

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Abstract A non-obese diabetic (NOD) mouse-derived embryonic stem (ES) cell line has been stably maintained in an undifferentiated state with a characteristic ES cell-like morphology, expressing the stem cell marker alkaline phosphatase, and displaying a normal diploid karyotype. After injecting the NOD-ES cells into blastocysts, chimeric mice were obtained. Small but significant numbers of lymphocytes expressed the NOD-derived MHC allele. When a chimeric mouse was mated with C57BL/6 mice, an agouti mouse was obtained, having the NOD-derived H-2 I-A^{g7}_{β} haplotype. Thus, an NOD-ES cell line which can differentiate into lymphocytes with potential for germ line transmission was successfully established.

Key words: Embryonic stem cell; Non-obese diabetic mouse; Germ line transmission; Diabetes; Insulitis

1. Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the preimplantation mouse embryo. ES cells can be stably propagated in an undifferentiated state in vitro using a feeder layer and/or leukemia inhibitory factor (LIF), and they also retain the ability to differentiate into all cell types found in embryonic and adult mice in vivo [1–5]. The genetic manipulation of ES cells by homologous recombination between the genomic locus and homologous DNA introduced into the cells has enabled the introduction of selected mutations into the mouse germ line [1–4]. The derived mutant mice have tremendously accelerated our understanding of the function of the targeted genes in vivo, and allowed the creation of mouse models for human diseases, clarifying the roles of specific genes in development, metabolic pathways and immunologic functions [1–4]. However, all the germ line competent embryonic stem cell lines in widespread use, such as ES-D3, CCE, E14, and AB1 [6–9], have been derived from blastocysts of the 129/Sv mouse strain. No ES cell lines derived from disease model animals have yet been reported. The ES lines derived from 129/Sv mice cannot yet be used directly for immunological and/or disease model studies, since they require extensive backcrossing to the desired mouse strain, which is very time-consuming and costly. Therefore the general use of ES cell technology remains limited in the immunologic field and animal models for human diseases.

The NOD mouse is a representative autoimmune animal model in which the development of insulitis is associated with the destruction of pancreatic β cells, thus leading to type 1 diabetes [10–12]. The development of autoimmunity in this animal model is controlled by multiple immune mechanisms [11,12] and many diabetes associated *idd* genetic loci (18 loci at present) have been reported [13,14]. Considerable evidence also suggests that cooperation between CD4 and CD8 T cells is required to promote the development of insulin-dependent diabetes mellitys (IDDM) in NOD mice and that islet β cell destruction is mediated by both CD4+ and CD8+ T cells [15,16]. Included among the effector cells of IDDM in NOD mice are CD4+ Th1 cells stimulated by interleukin-12 (IL-12), which preferentially secrete interferon-γ (IFN-γ) and tumor necrosis factor α, whereas the susceptibility to IDDM may be regulated by CD4+ Th2 cells, which preferentially secrete IL-4, IL-5, IL-6, IL-10 and IL-13 [12,17,18]. A high ratio of IFN-γ/IL-4 expression in islet infiltrating lymphocytes is predictive of both the onset of destructive insulitis and a high incidence of IDDM [19]. Thus, it is evident that lymphocyte functions play a major role in controlling the autoimmune diabetes in NOD mice. However, the exact role of each T cell subpopulation and/or cytokines in vivo still remains to be clarified. In order to address the specific role of genes and molecules of NOD mice in vivo, it is imperative to be able to perform genetic manipulation of NOD mice.

Only a few gene-targeted NOD mice have been reported, and these mice demonstrated the importance of MHC class I expression and B cells in disease pathogenesis, while IL-4 was found to be less important [20–23]. In these mutant mice, gene targeting was originally performed in 129/Sv mouse derived-ES cells, followed by backcrossing the gene-targeted mice to NOD mice in order to establish a congenic strain [20–23]. However, the possibility remains that the backcrossed mice are not completely congenic and may still lack disease-associated genes, since the autoimmune diabetes in NOD mice is known to be polygenic [13,14]. In addition, it is very time

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consuming and costly to perform backcrossing NOD mice, which must be performed for 10 generations to achieve a 99.9% probability that the backcrossed mice are congenic at all loci, requiring 3–4 years [24,25]. Even in speed congenic mice using marker-associated selection protocols, a total of five generations (four backcrossings) are needed, to achieve $<0.5\%$ donor genome contamination, requiring a period of 1.5–2 years [24,25]. Indeed, our group reported that a mild but apparent presence of insulitis in B cell-deficient NOD mice in eight times backcrossed mutant NOD mice [23], while another group reported a lack of insulitis and diabetes in B cell-deficient speed congenic NOD mice. Furthermore, another group reported that insulitis and diabetes in B cell-deficient NOD mice still remained [26]. Thus, the consequence of B cell deficiency in NOD mice remains somewhat controversial, thus suggesting that the backcrossed mice may not have the same genetic background. In addition, since the autoimmunity in NOD mice is dependent on the abnormal immune response mediated by the lymphocytes [12,13,15–18], it would be of potential importance to perform genetic manipulation of lymphocytes and observe the immunologic consequences of the immune system of NOD mice. This becomes possible by manipulation of lymphocytes controlling the disease development. To exclude the possible influence of undetermined genes in a congenic strain and to reduce the time and cost, the establishment of NOD mouse derived ES cells with a potential for germ line transmission has long been anticipated, although no NOD mouse-derived ES cell line has yet been reported.

Here we report the establishment of an ES cell line derived from NOD mice and its capability of differentiation into lymphocytes and of germ line transmission.

2. Materials and methods

2.1. Cell culture conditions

Dulbecco's modified Eagle's medium with high glucose (DMEM) supplemented with 20% fetal bovine serum (Gibco, prescreened for the ability to support the growth of ES cells), 50 U/ml of penicillin and 10 μg/ml of streptomycin (Gibco), with leukemia inhibitory factor (ESGRO) at a concentration of 10^4 U/ml, 5.5×10^{-5} M 2-mercaptoethanol solution, 0.5% of non-essential amino acid solution (Gibco), and nucleosides as described elsewhere [27] (complete medium) was used to isolate and propagate ES cells. A solution of 0.25% trypsin in 0.04% EDTA was used for routine subculture, in which the culture medium was discarded, warmed phosphate buffered saline (PBS) at 37°C was added to the cells, and then warmed EDTA-trypsin solution was added to the culture. Following 3 min of incubation, complete medium with fetal bovine serum was added and the cells were dispersed. The ES cells were incubated at 37°C in an atmosphere of 5% CO_2 in air.

2.2. Preparation of feeder layers

Primary embryonic fibroblasts were previously prepared from explanted 13–14 day BALB/c mouse fetuses. A confluent fibroblast culture was then treated with 10 μg/ml of mitomycin C (MMC) for 2 h and was washed three times with PBS. The MMC-treated fibroblasts were detached with EDTA-trypsin and 5×10^5 cells/ml were then seeded onto gelatin coated dishes (Sumilon, Japan).

2.3. Isolation of blastocysts

Between 3 days and 3.5 days after mating, the oviduct of pregnant NOD mice was flushed with complete medium using a 30 G needle and blastocysts were obtained. This procedure increased the number of eluting blastocysts that could be harvested.

2.4. In vitro characterization of ES cells

The established ES cells were stained for the presence of alkaline phosphate in the cytoplasm using the alkaline phosphatase staining kit (Muto Chem. Japan). For chromosome counts, ES cells at the 17th and 27th passage level were pretreated for 2 h with colcemid (0.1 μg/ml) and metaphase spreads were prepared and stained with Giemsa. Finally, the number of chromosomes was counted.

2.5. Generation of injection chimeras

Host embryos were recovered at the morula stage from the oviducts of hormonally treated C57BL/6 females and cultured overnight. Blastocysts were transferred together with suspended ES cells at the 24th passage level into drops of injection medium. About 10–20 ES cells were injected into each blastocyst. After a recovery period of about 2 h, the injected blastocysts were transferred into the uterine horn of pseudopregnant ICR foster mothers. Chimeric offsprings were identified by a mixed white albino coat color on a black background. Chimeric male mice were further mated to C57BL/6 mice to obtain an agouti mouse.

2.6. PCR for detection of NOD-specific I-A^{g7}_{β} genes

I-A genes of the class II molecules were determined as previously described [20]. PCR for genomic DNA was performed by using primers with additional *Eco*RI sites specific for I-A^{g7}_{β} (5′-A^{g7}_{β}: 5′-TCTAGAATTCACAGCGACGTGGGCGAGT-3′ and 3′-A^{g7}_{β}: 5′-TCTAGAATTCCGTAGTTGTGTCTGCACG-3′). The I-A_{β} genes were amplified as follows: denaturing at 94°C for 1.5 min, annealing at 55°C for 1.0 min and extension at 72°C for 1.0 min, for 25 cycles on a DNA thermal cycler [20,23].

2.7. Flow cytometric analysis

Peripheral blood was taken from the periorbital sinus, followed by lysis of red blood cells with ammonium chloride. For flow cytometric analysis, peripheral blood leukocytes of mice were first treated with 1 μg/10^6 cells of rat anti-mouse CD16/CD32 (Fcγ III/II receptor) monoclonal antibody (PharMingen, San Diego, CA, USA) to reduce Fc receptor-mediated non-specific binding of following antibodies. Then, the cells were stained with 0.5 μg/10^6 cells of biotin-labeled anti-H-$2K^d$ (SF1-1.1) (PharMingen), or with 1.0 μg/10^6 cells of fluorescein isothiocyanate (FITC)-labeled anti-H-$2K^b$ (AF6-88.5) (PharMingen) mAb. The cells were washed with PBS containing 2% fetal bovine serum and 0.1% NaN_3, followed by 0.5 μg/10^6 cells of streptavidin-Red 670 (Gibco, Grand Island, NY, USA) staining. The cells were analyzed by a flow cytometer (Becton Dickinson).

3. Results

3.1. Isolation of ES cells

In the first series of experiments, four ES cell lines were

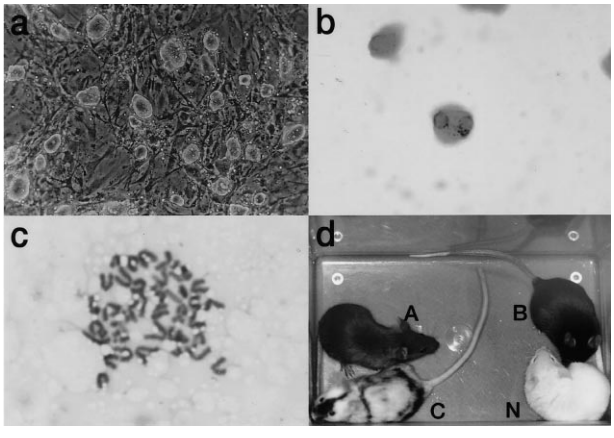

Fig. 1. NOD mouse-derived ES cell line, formation of chimeric mice and germ line transmission. a: Growing colonies of the established ES cell line derived from a NOD mouse (2 day culture) (×100). b: Alkaline phosphatase staining (×400). c: A representative karyotype of the NOD mouse-derived ES cell line (×1000). d: Mice derived from the NOD ES cell line. A: agouti mouse, germ line transmission, B: control C57BL/6 mouse, C: chimeric mouse, N: control NOD mouse.

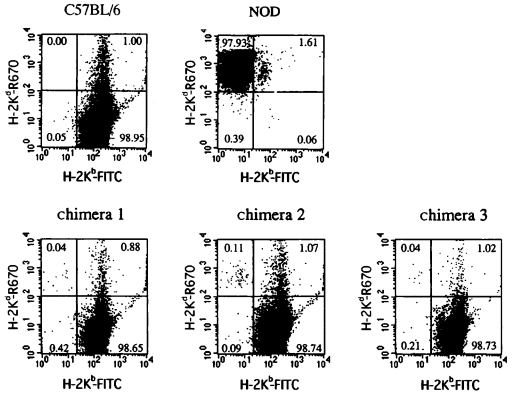

Fig. 2. Flow cytometric analysis of peripheral lymphocytes in chimeric mice. A low percentage of peripheral blood lymphocytes of chimeric mice expressed H-2K^d without H-2K^b, a surface phenotype that is derived from the NOD ES cells.

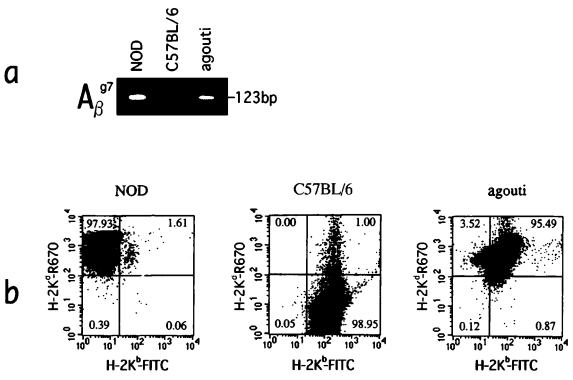

Fig. 3. a: PCR detection of the NOD mouse-derived H-2 I-A_β^{g7} gene in the peripheral lymphocytes of an agouti mouse. b: Flow cytometric analysis of the peripheral lymphocytes of an agouti mouse revealed the coexpression of both H-2K^b and H-2K^d class I molecules.

isolated from 138 blastocysts. Three lines lost the ES-like characteristics during further passage. The remaining cell line morphologically resembled ES cells, however, it had developed a tetraploid karyotype, and was thus abandoned. In the second series of experiments, 181 blastocysts were cultured, and one cell line exhibited the characteristic morphology of ES cells (Fig. 1a), and expressed a stem cell marker, alkaline phosphatase in its cytoplasm (Fig. 1b). More than 90% of the ES cells possessed 40 chromosomes with a Y chromosome, 21/23 (91%) at the 17th and 33/35 (94%) at the 27th passage, and the cell line displayed a normal diploid karyotype (Fig. 1c). The cell line was stably maintained in a morphologically undifferentiated state for over 27 passages.

3.2. Production of chimeric mice and differentiation into lymphocytes in vivo

Using the NOD mouse-derived cell line at the 24th passage level, we obtained 22 chimeric mice (nine males, 13 females) (Fig. 1d) out of 90 offspring. Based on skin condition three (one male, two females) of 22 mice expressed more than 80% chimerism (Fig. 1d), six (one male, five females) showed 50–80% chimerism, and six (four males, two females) showed 30–50%. Seven (two males, five females) showed less than 30% (Table 1). To assess whether the ES cells could differentiate into lymphoid tissue, we analyzed the peripheral blood lymphocytes of chimeric mice expressing over 80% skin chimerism. A small but significant number of peripheral blood lymphocytes expressed H-2K^d but not H-2K^b in all chimeric mice examined (Fig. 2), indicating that ES cells derived from NOD mice maintain the potential to differentiate into lymphocytes.

3.3. Germ line transmission

The highly chimeric male mouse (more than 80%) was mated with C57BL/6 female mice. One out of 97 offspring was agouti in appearance (Fig. 1d). Genomic DNA analysis of the agouti mouse by PCR revealed the presence of the H-2 I-A_β^{g7} gene, which was derived from the NOD mouse (Fig. 3a). In addition, a flow cytometric analysis showed coexpression of both H-2K^b and H-2K^d class I molecules (Fig. 3b), thus suggesting that the ES cell DNA was transmitted through the germ line.

Table 1
Formation of chimeric mice from the NOD mouse-derived ES cell line

Skin chimerism	Number of chimeric mice
≧80%	3 (♂ 1, ♀ 2)
50–80%	6 (♂ 1, ♀ 5)
30–;50%	6 (♂ 4, ♀ 2)
< 30%	7 (♂ 2, ♀ 5)
Total	22 (♂ 8, ♀ 14)

4. Discussion

We successfully established an ES cell line with a potential for germ line transmission. Peripheral blood of chimeric mice contained lymphocytes derived from the NOD ES cell line, indicating that it could differentiate into lymphoid tissues. ES cell technology has greatly contributed to advances in analysis of in vivo function of genes [1–4]. For that purpose, pluripotent ES cells derived from 129/Sv mice have been widely utilized, but no ES cell line derived from a disease model animal has been reported. This has hampered the overall use of ES technology in animal models for human diseases. ES cells derived from more well-studied mouse strains could hasten investigation of in vivo gene function. In order to apply the ES cell technology in the immunologic field, Ledermann and Burki reported the establishment of a germ line-competent ES cell line derived from the C57BL/6 mouse [5]. They used a preconditioned medium obtained from 5637 human bladder carcinoma cells and the explanted embryonic cells of CD-1 mice as a feeder layer. LIF has been reported to be capable of replacing the feeder layers of embryonic fibroblasts and conditioned medium [28,29]. Independently of the approach chosen, the establishment of embryonic stem cell lines largely depends on the establishment of optimal culture conditions [5–9]. In the present study, we used both an ES medium containing 1×10^4 u/ml of LIF and embryonic fibroblasts derived from BALB/c mice as a feeder layer. These culture conditions were essential for supporting the growth of the NOD mouse-derived ES cells, while also maintaining them in an undifferentiated state. When deprived of either LIF or the feeder layer, the ES cells tended to begin to differentiate into epithelial and other tissues, thus emphasizing the importance of the culture conditions for isolating and propagating the estab-

lished ES cell line from NOD mice. The NOD ES cell line had a modal chromosome number of 40 with a Y chromosome, indicating a male genotype. This genotype is consistent with that of previous ES cell lines, which are predominantly male, probably due to chromosome instability [5–9].

In the present study, we described a NOD mouse-derived ES cell line which is competent for differentiation into lymphoid tissue in chimeric mice. Since lymphocytes are imperative for the development of autoimmune diabetes in NOD mice, this cell line will be useful for the genetic manipulation of lymphocytes controlling the disease. The roles of the manipulated lymphocytes can be investigated by transferring them into immunodeficient NOD mice, such as T cell-deficient, severe combined immunodeficient (SCID), or B cell-deficient NOD mice to clarify the in vivo role of genes and molecules of lymphocytes responsible for disease development. In addition, the germ line transmissible NOD ES cells may also be useful for the direct genetic manipulation of the NOD mouse by homologous recombination strategies, or may further be useful for the conditional gene knockout using the Cre-loxP system [30]. Although the NOD ES line successfully exhibited germ line transmission, the efficiency was low. Similarly, although a germ line-competent ES cell line has been successfully established from C57BL/6 mice, the transmissibility was reported to be low, possibly due to a diminished capacity to differentiate into sperm [5]. Only a few ES cell lines are able to effectively transmit to the germ line except for ES lines derived from 129/Sv mice, which are characterized by a high incidence of spontaneous testicular teratomas or teratocarcinomas [27]. It is thus possible that the differentiation potential of germ cells derived from ES cells of mice other than 129/Sv mice might be inherently lower. We are now subcloning the ES cell line based on the morphological characteristics with clear margins, and we have thus obtained four sublines. An attempt to find an ES cell line with increased germinal transmission capability is also currently under way.

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