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Studies on the Generation of Transgenic Cow Producing Human Lactoferrin in the Milk

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락토페린을 우유에서 생산하는 형질전환 젖소의 개발에 관한 연구

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요 약

본 연구는 인체 락토페린(hLF)을 우유 중으로 생산하는 형질전환 젖소의 개발에 관한 것이다. 이를 위한 모델 시스템으로서 락토페린 cDNA와 소의 베타-카제인 프로모터를 이용하여 형질전환 생쥐를 개발하였다. 발현 벡터의 락토페린에 대한 발현효율을 증가시키기 위하여 2개의 재조합 인트론을 삽입하였다. 20계통의 형질전환 생쥐를 개발하였는데 유즙에서의 락토페린 발현량은 1~200μg/ml이었다. hLF RNA의 발현 양상을 유선조직을 포함하여 뇌, 신장, 간 조직 등에서 조사하였을 때, 오직 유선에서만 발현되었을 뿐 아니라 엑손/인트론 경계 부위에서 정확하게 splicing되었다. hLF를 생산하는 형질전환 젖소를 개발하기 위하여 위에서 기술한 DNA를 소의 수정란에 미세주입한 후, 외과적 또는 비외과적 방법으로 대리모에 이식하였다. 한편, DNA가 주입된 수정란의 상태가 임신율에 미치는 영향을 조사하였다. 수정란을 최우수, 우수, 보통 등 3등급으로 나누었을 때, 각각의 임신율은 38.9, 15.4, 14.3%로 나타났다. 현재까지 유전자가 주입된 수정란을 대리모에 이식하여 태어난 35마리의 송아지 중, 30마리는 형질전환되지 않았으며 나머지는 현재 분석 중에 있다. 이상의 결과로 본 연구자들은 DNA가 미세주입된 젖소 수정란의 배양과 이식에 필요한 제반 기술을 확립하였으며, 아울러 임신율에 영향을 주는 여러 인자들에 대한 연구도 함께 조사하였다.

I. INTRODUCTION

Transgenic animals which experess foreign proteins in their mammary glands have been useful tools for the mass production of human proteins (Hennighausen, 1992). DNA sequences conferring mammary-specific expression to the

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foreign genes of interest have been derived from the promoters of major milk protein genes such as whey acidic protein (Gordon et al., 1987; Pittius et al., 1988), β-lactoglobulin (Archibald et al., 199), α-casein (Meade et al, 1990), β-casein (Lee et al., 1988; Kim et al., 1994) and α-lactalbumin (Stinnakre et al., 1991). In most studies, the foreign proteins were synthesized and secreted into the milk although in some cases the expression of the transgene was not restricted to the mammary gland. The advent of such technology has prompted researches of using transgenic animals as bioreactors. Pharmaceuticals and other useful human proteins such as human α_1-antitrypsin (Archibald et al., 1990; Carver et al., 1992), tissue plasminogen activator (Gordon et al., 1987; Ebert et al., 1994), human serum albumin (Barash et al., 1994), and human hemoglobin (Sharma et al., 1994) were produced in transgenic mouse, rat, sheep, goat, and pig. Although very few transgenic systems have been described where the expression level of the transgene approached that of the endogenous gene, many of them proved to be successful by expressing more than a gram of transgene product per liter of milk. Lactoferrin (LF) is an iron binding protein in milk and a member of transferrin family (Brock, 1985). Human lactoferrin (hLF) is a glycoprotein consisting of a single polypeptide chain (~80 kDa) containing 691 amino acids (Rey et al., 1990). The levels of hLF can reach up to 6 g/l in colostrum and decrease to 1 g/l in mature milk. The levels of LF in bovine milk, however, are lower ranging from 0.02 to 0.2 g/l (Reiter, 1978). Two studies have been reported for the mass production of hLF. One is about the filamentous fungi *Aspergillus orizae* in which up to 25 mg/l of hLF was expressed (Ward et al., 1992). The other study exploited transgenic mice system in which regulatory sequences of the bovine α_{S1}-casein gene were used to target hLF expression into the mammary glands (Platenburg et al., 1994).

We have generated transgenic mice carrying human lactoferrin cDNA (Kim et al., 1994). Regulatory elements of the bovine β-casein gene were used to control the expression of hLF cDNA. We have followed the transmission and expression of hLF in eleven murine lines.

Ⅱ. RESULTS

1. Generation and transmission of transgenic mice

To date, eleven line have been generated as transgenics. Ten lines appeared to inherit the transgene stably through several generations. One line (#15) failed to give birth to offspring. Five of the eleven founder animals were females, and six were males. These were used in breeding program to follow inheritance and expression of the transgene from generation to generation. G0 animals were mated with F1 (C57BL/6 X DBA) to get transgenic progeny. As shown in Table 1, the transmission rate of transgenicity varied from 10 to 50% implying that some lines propagated in a Mendelian fashion whereas other lines as germ line mosaic. The founder animals and offspring were analyzed for copy number, inheritance, and expression of the transgene. Copy numbers ranged from 1 to 38 (Table 1).

2. Analysis of hLF RNA

RT-PCR was carried out to determine the 5′ and 3′ ends of transgene RNA. When sets of primers, (1, 3) and (4, 5) of which ends were complementary to the 5′ and 3′ end of the expected RNA, were used for RT-PCR, they produced correct-sized products (lanes 2 and 4, Fig. 1).

Table 1. Analysis of transgenic founder mice

Founder	Sex	No. of G1 Transgenic /Progeny	Germ line transmission rate (%)[a]	Copy no.
1	M	1/10	10.0	25
2	F	1/9	11.0	1
3	F	3/9	33.3	18
6	M	6/14	42.9	38
7	M	2/10	20.0	13
8	M	1/10	10.0	4
9	M	3/8	37.5	8
10	M	4/10	40.0	23
11	F	2/6	33.3	9
15	F	0/3	0	ND
20	F	3/6	50.0	15

The sex of animals is indicated by "F" for female and "M" for male.

[a] Percentage is the number of transgenic animals per live births.

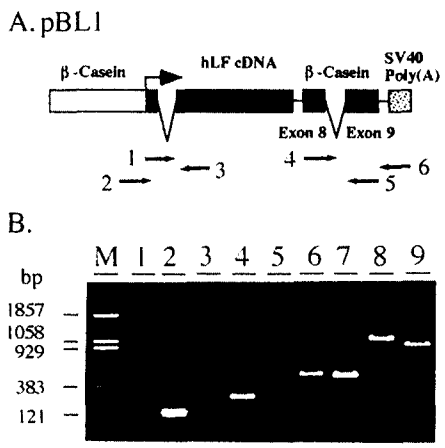

Fig. 1. RT-PCR analysis of total RNA from the mammary gland of transgenic mouse. (A) Schematic diagram of the recombinant hLF cDNA microinjectd into mouse embryos. Open box is regulatory sequences of bovine β-casein gene. Black boxes indicate hLF cDNA sequence and exons 8 and 9 of bovine β-casein gene. SV40 polyadenylation signal is shown as a stippled box. Transcription initiation site and primers used for RT-PCR are indicated as arrows. (B) Lanes 1~4 are products of RT-PCR from RNA of mammary gland and 5~8 are PCR products of injected DNA. Sets of primers for the PCR analysis are (1, 3) for lanes 1 and 6 (2, 3) for lanes 2 and 7 (4, 6) for lanes 3 and 8 (5, 6) for lanes 4 and 9. No RNA was added for lane 5. Lane M is a pBR322 DNA molecular weight marker digested with BstNI.

However, primer sets (1, 3) and (4, 6) of which ends were complementary to the 5′-flanking sequence (−2) to −1 from the transcription start site of bovine β-casein gene) or to the 3′-flanking sequence just downstream of SV40 polydenylation site, did not produce any PCR product (lanes 1 and Fig. 1). These results show that the recombinant hLF RNA has the complete coding seuqences starting from the transcription initiation site of the bovine β-casein gene and ending at the SV40 polyadenylation region.

The transgene message was further analyzed by northern blot hybridization. For this experiment, total RNAs from various tissues of transgenic and normal mice were isolated and hy-

bridized with two kinds of probes. One probe was the 5′-side EcoRI-SmaI fragment (1.2 Kb) of hLF cDNA. This probe strongly hybridized with RNAs of the mammary glands of transgenic mice (lanes Mg and Mg*, Fig. 2A).

Signals of less intensity were also detected in RNAs from the liver of transgenic mice and the mammary gland of normal mice (lanes Li and Mg⁻, Fig. 2A). When the same filter was hybridized with a transgene-specific DNA probe spanning exons 8 and 9 of the bovine β-casein cDNA, only RNAs from the mammary glands of transgenic mice were hybridized (lanes Mg and Mg*, Fig. 2B). This means that the transgene was exclusively expressed in the mammary gland of transgenic animals. The band appeared on the liver RNA of the transgenic mouse in Fig. 2A may be due to a cross-hybridization of the endogenous mouse lactoferrin message with hLF sequences.

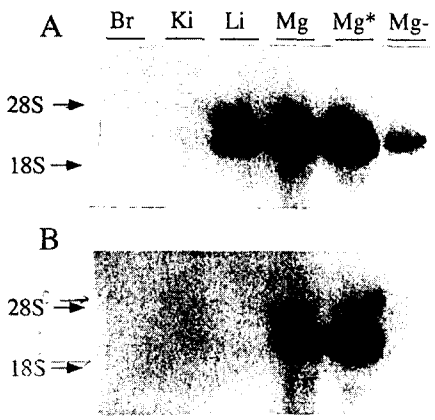

Fig. 2. Northern blot analysis of total RNA. RNAs isolated from mouse tissues were size-fractionated on an agarose gel, transferred to a nylon membrance and probed with (A) hLF cDNA and (B) exons 8 and 9 of bovine β-casein cDNA. Tissues analyzed were brain (Br), kidney (Ki), liver (Li) and mammary gland (Mg) of G2 of the transgenic line #2. Mg* is mammary gland of G0 of transgenic line #2. Mg⁻ is mammary gland of normal mluse. 28S and 18S ribosomal RNAs are indicated.

3. Characterization of hLF in milk of transgenic mice

Milk was collected from female mice on day 10 of lactation. The amount of milk ragned from 500 to 1,000 μl per one lactation per one mouse. Of the ten established transgenic lines, milk could be collected from six lines. To date, four lines have stably expressed hLF in their milk. To characterize the recombinant hLF in the milk, western blot analysis was carried out by fractionating the milk proteins on an SDS-polyacrylamide gel. A polyclonal rabbit anti-hLF was used to detect hLF.

As shown in Fig. 3, two bands appeared in the milk of transgenic mouse. The 80 kDa protein band specifically appeared in the milk of trans-

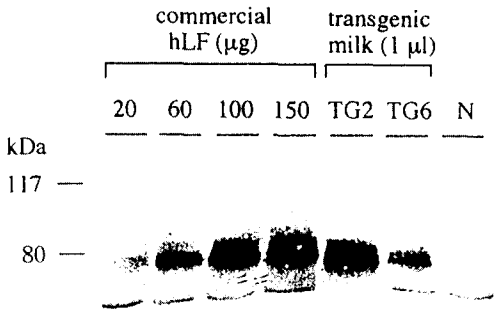

Fig. 3. Immunoblot analysis of hLF in milk of trnasgenic mice one μl of whey fractions of milk collected from the transgenic lines #2 and #6 were loaded onto an SDS-polyacrylamide gel and analyzed by immunoblotting. 20, 60, 100 and 150 μg of commercially available hLF were also analyzed to compare the concentrations.

genic mouse and its size was identical to that of the known hLF. The smaller 78 kDa band also appearing in the milk of normal mouse, might be the result of the non-specific reaction by the polyclonal antibody with a milk protein.

The expression level of hLF in milk was determined by ELISA. Table 2 summarizes the expression level of hLF at different generations and lactations of transgenic lines. The G0 mice of lines #2 and #20 were females and their expression leveles were 19~200 μg/ml. Their transgene copy numbers, as determined by slot blot analysis, were about one and sixteen, respectively (see Table 1). The G0 mice of lines #6 and #9 were males, and therefore, their expression of hLF was determined from the G1 female mice. Line #6 expressed 1~13 μg/ml of hLF. Two lines (#3 and #9) did not express any detectable amount of hLF although they carried 18 and 8 cpoies of the transgene in the genome. From the results in Table 1 and 2, it is clear that ther is no apparent relationship between copy numbers and expression level.

4. Transfer of DNA-injected bovine embryos

Cleavage rate to 2- to 8-cell stage and developmental rate of the embryos to blastocyst stage was 72.0 and 5.2%, respectively (Table 5). Out of 68 embryos transferred to 51 recipients by surgical or non-surgical method, 7 calves were normally born (Table 6). But it was shown by PCR and southern blot that there was no transgenic animals. All transferrable balstocysts developed from DNA-injected zygotes were individually classified into 3 developmental stages (early, mid, expanded blastocysts) and then individually transferred to recipients. The more developmental stage of DNA-injected blastocysts was advanced, the more pregnancy rate after transfer was increased. Thus, there was difference in the pregnancy rates of DNA-injected bovine blastocysts among the developmental

Table 2. hLF expression in founder transgenic mice and their progeny

Line	Generation			hLF content in milk of lactation[a]
	G0	G1	G2	
2	2			19
			2-3-1	2
3	3			0
		3-1		0
		3-2		0
6		6-1		13
9		9-3		0
20	20			200

Table 3. Microinjection of pBL1 into IVF-derived bovine eggs

No. of embryos injected	No. of embryos survived (%)[a]	No. of embryos cleaved (%)[b]	No. of embryos developed to bastocysts (%)[c]
3,370	2,516 (74.6)	2,035 (80.9)	302 (16.7)

[a] : No. of embryos survied /No. of embryos injected × 100

[b] : No. of embryos cleaved /No. of embryos survived ×100

[c] : No. of blastocysts /No. of embryos survived × 100

Table 4. Transfer of DNA-injected bovine embryos

Group	No. of embryos transferred	Pregnant /Recipients	Abortion	Calves	
Fresh	77	12/51	5	7	♂ : 6 ♀ : 1

Table 5. Effect of developmental stages of DNA-injected bovine embryos on pregnancy rate

Developmental stage	No. of embryos transferred	Pregnant /Recipients	Pregnant rate (%)	Abortion	Calves
Expanded	15	6/15	40.0	2	4
Mid	20	4/20	20.0	1	3
Early	16	2/16	12.5	2	0
Total	51	12/51	23.5	5	7

Table 6. Effect of quality of DNA-injected blastocysts on pregnancy rate

Embryo quality	No. of embryos transferred	Pregnant /Recipients	Pregnant rate (%)	Abortion	Cavles
A	18	7/18	38.9	3	5
B	26	4/26	15.4	2	1
C	7	1/ 7	14.3	0	1
Total	51	11/51	23.5	5	7

A : excellent, B : good, C : fair

stages. Expanded blastocysts (40.0%) had significantly higher pregnancy rate than those of early (12.5%) and mid blastocysts (20.0%) (Table 7).

Effect of embryo quality of DNA-injected blastocysts on pregnancy rate after transfer was investigated. All blastocysts developed from DNA-injected zygotes were individually classified into 4 grades (excellent, good, fair, poor) of embryo quality by morphological appearence and the embryos with poor quality were then discared. The other blastocysts except poor quality were individually transferred to the uterus of recipients by surgical or non-surgical method. Higher pregnancy rate of (38.9%) DNA-injected embryos was shown in excellent embryos. Pregnancy rates in the groups of good and fair embros were 15.4 and 14.3%, repectively (Table 8). Effect of culture period of DNA-injected bovine embryos on pregnancy rate after transfer was investigated. When Day-6 blastocysts of culture were transferred, there was no pregnancy.

Table 7. Effect of culture period of DNA-injected bovine embryos on pregnancy rate after transfer

Day of culture	No. of embryos transferred	Pregnant /Recipients	Pregnant rate (%)	Abortion	Calves
Day 6	11	0/11	0	0	0
Day 7	28	8/28	28.6	4	4
Day 8	12	4/12	33.3	1	3

Pregnancy rates of Day-7 and -8 blastocysts were 28.6 and 33.3%, respectively (Table 9). There was no difference on pregnancy rate between Day-7 and -8 bovine blastocysts after DNA injection. Thus, we established the techniques for transfer and culture of DNA-injected bovine embryos. In addition, factors affecting the pregnancy rate of DNA-injected embryos after transfer were investigated.

Ⅲ. SUMMARY

Human lactoferrin (hLF) was expressed in the mammary gland of transgenic mice. Expresion of hLF was achieved by palcing its cDNA under the control of bovine β-casein gene. To improve the hLF expression level, two artificial introns were introduced into the expression vector. One intron is a hybrid-splice consisting of bovine β-casein intron 1 and rabbit β-casein intron Ⅱ. The other intron is a DNA fragment spanning intron 8 of bovine β-casein gene. Transgenic mice were developed which expressed hLF in their milk. Twenty lines of transgenic mice were produced. hLF was present in the milk at concentrations of 1~200 μg/ml. hLF RNA was only detected in the mammary gland of transgenic mice. The expressed RNA was correctly spliced at the exon/intron junctions. To generate transgenic cows secreting active hLF in their milk, we transferred the DNA-injected bovine embryos to recipient heifers by surgical and non-surgical methods out of 68 embryos transferred to 51 recipients by surgical or non-surgical method, 7 calves were normally born. Effect of embryo quality of DNA-injected blastocysts on pregnancy rate after transfer was investigated. Higher pregnancy rate of (38.9%) DNA-injected embryos was shown in excellent embryos. Pregnancy rates in the groups of good and fair embryos were 15.4 and 14.3%, respectively. Effect of culture period of DNA-injected bovine embryos on pregnancy rate after transfer was investigated. When Day-6 blastocysts of culture were transferred, there was no pregnancy. Pregnancy rates of Day-7 and -8 blastocysts were 28.6 and 33.3%, respectively. There was no difference on pregnancy rate between Day-7 and -8 bovine blastocysts after DNA injection. Thus, we established the techniques for transfer and culture of DNA-injected bovine embryos. In addition, factors affecting the pregnancy rate of DNA-injected embryos after transfer were investigated.

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