We found that stem-cell leukemia (SCL), also known as T cell acute-lymphocytic leukemia (Tal-1) gene expression, was upregulated in the maturing rat testis. Strong expression of Tal-1 was detected in the normal maturing rat testis by Northern blotting. Western blotting revealed the protein size to be about 34 kDa. Protein expression was wide-spread in spermatocytes, spermatids and spermatagonia in accordance with the seminiferous epithelium cycle, as determined by an analysis of immunohistochemistry. Gene expression of Tal-1 regulatory gene, NKX3.1, was negatively correlated with Tal-1 expression. Human Tal-1 expression in the maturing testis as well as in bone marrow was observed, which suggests that the gene product is a novel cancer-testis antigen candidate. Taken together, Tal-1 may be involved in cell division, morphological changes, and the development of spermatogenic cells in the normal rat testis.

Key words: immunohistochemistry; in situ hybridization; spermatogenesis; stem-cell leukemia; T-cell acute-lymphocytic leukemia

Spermatogenesis is regulated by the expression of many genes, and morphological changes in spermatogenic cells and their maturation proceed systematically during this process. We have cloned and characterized some of these genes by differential display (DD), and we categorized them according to expected functions. Of these, tumor antigens commonly expressed in normal testis are interesting, because they can be used for cancer diagnosis, and are promising tools for cancer immunotherapy, and can be used to understand the process of spermatogenesis. Colon cancer specific antigens have been screened by a recombinant cDNA expression library (SEREX) from an individual cancer patient, and they are promising candidates as cancer-testis (CT) antigen genes. We have identified the rat homolog of the serologically defined colon cancer antigen 8 gene (Sdccag8), which was screened by SEREX, and expression was rat testis specific. The gene product was identified as serologically defined colon cancer antigen 8 (SDCCAG8) and centrosomal colon cancer autoantigen protein (CCCAP), and its C-terminal portion was identical to the serologically defined human colon cancer autoantigen (NY-CO-8). According to this result, we consider it a rat CT antigen candidate.

T-cell acute-lymphocytic leukemia (T-ALL) is a malignant blood stem-cell cancer commonly observed in children. In T-ALL, blood stem cells do not differentiate into mature blood cells, such as erythrocytes or white blood cells, and patients have severe anemia, are vulnerable to infectious diseases, and usually have a poor prognosis. Novel CT antigen candidates are therefore promising tools for diagnosing T-ALL. One such candidate, TSGA10, has been reported. It might be involved in the mitotic checkpoint during T-ALL development. We are currently searching for other leukemia CT antigens, and recently studied the expression of the T-cell acute-lymphocytic leukemia (Tal-1) and the stem-cell leukemia (SCL) gene in maturing rat testes. Baer identified a Tal-1 gene rearrangement in T-ALL patients due to chromosomal translocation and DNA recombination.

Normal Tal-1 expression in mouse embryogenesis has been observed in the yolk sac blood island, and in the fetal liver, spleen, and thymus, and in developing vascular system, brain, and cartilage. Tal-1 deletion mutants suggest that the gene functions in angiogenesis and hematopoietic differentiation, such as bone marrow erythropoiesis during embryogenesis, as well as in T-ALL development. TAL-1 also functions with GATA1 and LIM transcription factors during erythropoiesis, and is an important basic helix-loop-helix (bHLH) transcription factor expressed in developing erythroid cells. Chromatin immunoprecipitation (ChiP) sequencing analysis has been used to identify TAL-1 target genes, which include tumor suppressor genes such as NKX3.1, stem cell regulators such as Gfi1, and endothelial specific adhesion molecules such as VE-cadherin. The TAL-1 complex binds the E-box-associated GATA motif, and this interaction can modify gene expression during angiogenesis and blood cell development. A combination of TAL-1 and LIM domain protein LMO2 has been found to lead to the development of T-ALL.

Human Tal-1 expression is largely limited to the fetal liver and to adult bone marrow, brain, and cartilage.
TAL-1 protein expression in tissues and cell lines has been analyzed, but expression in the developing testis is not determined. To evaluate the importance of TAL-1 expression during testis development, we examined it in the maturing rat testis and determined its function in relation to spermatogenesis.

Materials and Methods

Semi-quantitative RT-PCR and Northern blotting. Sprague-Dawley (SD) rats were purchased from CLEA Japan (Tokyo). Total RNA from testes at various developmental stages and from individual organs of 9-week-old male rats and a female rat was prepared using TRIzol reagent (Life Technologies, Carlsbad, CA) following the manufacturer’s instructions. A bone marrow cell suspension was harvested by flushing the cavity of the femurs and tibiae with phosphate-buffered saline, the cells were collected by centrifugation, and total RNA was prepared. Human total RNA was purchased (Takara Bio, Shiga, Japan). Fetal liver RNA was prepared from 63 spontaneously aborted male and female Caucasian fetuses (ages 22–40 weeks); liver RNA was from a 51-year-old male Caucasian, bone marrow RNA was from 56 Asian males and females (ages 22–95); and testis RNA was from five Asians (ages 21–29). cDNA was synthesized from 1 μg of RNA using a first-strand cDNA synthesis kit including Super Script III Reverse Transcriptase (Life Technologies). PCR was performed using Takara Roche Es Taq DNA polymerase (Takara Bio) under the following conditions: 94°C for 5 min, then 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 5 min; followed by extension at 72°C for 10 min. The PCR amplification primers were as follows: rat Tal-1 sense primer 5'-GGT-TGGAATATGTGGGACAGTAAC-3' and antisense primer 5'-AAC-ACTTGTCAGGGAATACCTCC-3' (accession no. NM_001107958), rat Nkx3.1 sense primer 5'-TCCTGCTTGACGAGCTCGAGGA-3' and antisense primer 5'-AGCTGCTGACAGCAGGAC-3' (accession no. NM_001341144), rat Gfi1 sense primer 5'-GGCAAGATGGGTGACCG-3' and antisense primer 5'-GCGCTTGAAACCA-TGTGTCCTTC-3' (accession no. NM_012566), rat VE-cadherin sense primer 5'-CCATATTGGGAAAGATACCAAGTCC-3' and anti-sense primer 5'-TCTCTGGCTTCCCACACGATCTC-3' (accession no. NM_001107940), and human Tal-1 sense primer 5'-CAAGGCTCTGA-GGTGCTCTT-3' and antisense primer 5'-GGACAGGGTTTGGTC-TCAATTCCG-3' (accession no. NM_003189). Mammalian Gapdh primers were used for PCR amplification as internal control. The PCR products were analyzed by 1.5% agarose gel electrophoresis. For Northern blotting, 5 μg of RNA was electrophoresed in denaturing gel containing formaldehyde and blotted onto a Hybond N+ nylon membrane (GE Healthcare, Buckinghamshire, UK). The Tal-1 cDNA probe was labeled by a random primer labeling system (GE Healthcare) using [α-32P] dCTP. The membrane was hybridized with the probe, then washed with buffer containing 1 x SSC and 0.1% SDS at 65°C for 20 min, and radioactivity was measured using a FLA-5000 fluorescent image analyzer (GE Healthcare). It was rehybridized with rat Gapdh cDNA as internal control to confirm RNA integrity and quantity.

Cloning and sequencing of the Tal-1 upstream region. The rat Tal-1 cDNA sequence was deposited in the DNA database under accession no. NM_001107958. It encodes a predicted 212-amino-acid protein, but the upstream sequence of the proposed start methionine codon was different from that of mouse and human. To clarify the upstream sequence, we designed primers for PCR amplification in a 5’ direction from the proposed start codon based on conserved sequences of mouse and human. The designed upstream sequence primer was 5'-GGACCT-TCACGGCAACGCTAAG-3', and the downstream sequence primer was 5'-GCTAAAGGCTTATAGACCGCCG-3'. PCR was performed using Tks Gfles DNA polymerase (Takara Bio) according to the following protocol: 94°C for 1 min, then 34 cycles of 94°C for 10 s, 60°C for 15 s, and 68°C for 1 min, followed by extension at 68°C for 10 min. Amplified cDNA, in which A-overhangs were introduced, was cloned into pGEM-T Easy vector and sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Tokyo). The DNA sequence was compared to that of Tal-1 cDNA sequences deposited in the DNA database.

In situ hybridization (ISH). A riboprobe designed using the same sequence as the rat Tal-1 RT-PCR amplified fragment was synthesized using a DIG RNA Labeling kit (Roche, Basel, Switzerland). A 5-μm section of 8-week-old rat testis was hybridized with the probe, and the signal was developed using a DIG Nucleic Acid Detection Kit (Roche) following the manufacturer’s instructions. The sections were counterstained with methyl green.

Western blotting and immunohistochemistry. Rat tissue samples were prepared and purified using Complete Lysis-M (Roche Applied Science, Tokyo), and 10 μg of each protein sample was electrophoresed on a sodium dodecyl sulfate 10% polyacrylamide gel (SDS–PAGE), and transferred to a Hybond C membrane (GE Healthcare). The membrane was hybridized with Tal-1 antisera (QC1680, Aviva Systems Biology, San Diego, CA) (1:2,000) and incubated with HRP-conjugated goat anti-rabbit IgG (Cosmo Bio, Tokyo) (1:10,000) as secondary antibody. Bound antibody was detected using Immunoblot Western Chemiluminescent HRP Substrate (Millipore, Tokyo). Rabbit polyclonal anti-GAPDH (Cosmo Bio) was used as control primary antibody (1:2,000). The sections used in ISH were hybridized with the same primary antisera (1:100) as used in Western blotting, and the signals were detected using a Histoline DAB substrate kit (Nichirei, Tokyo) following the manufacturer’s instructions.

Results

RT-PCR, Northern blotting, and Western blotting

We used DD to screen for genes expressed specifically during the sexual maturation of the rat testis, and focused on one candidate, Tal-1 (SCL). Gene expression was determined by semi-quantitative RT-PCR and Northern blotting. Tal-1 gene expression was observed as a 458 bp specific band in RT-PCR and a 28S rRNA-size band in Northern blotting. Expression was upregulated during testicular development, reaching maximum levels in 7-week-old and older rats (approximately a 6-fold increase from 3 weeks to 7 weeks of age). Strong expression of Tal-1 was detected in the maturing rat testis, and expression in the normal bone marrow was about one quarter the intensity of the testis according to the Northern blotting results, although faint expression was observed in the lung, heart, and cerebral of the 9-week-old rats by RT-PCR (Fig. 1A, B). Tal-1 protein expression was determined by Western blotting, and was found to be weak in the testes of the 3-week-old rats, increasing after 7 weeks of age and remaining stable thereafter. The expression level in the testis was almost same as that of the bone marrow. The protein size was approximately 34 kDa (Fig. 1C).

TAL-1 regulatory gene expression was measured by semi-quantitative RT-PCR. Although Gfi1 expression was thymus-specific and VE-cadherin expression was wide spread among the mature rat organs, Nkx3.1 expression was testis-specific, and the level was highest in 3-week-old and decreased thereafter (Fig. 2A). The expression of human Tal-1 was high in the fetal liver, adult bone marrow, and testis, but expression in adults even in the liver was weak (Fig. 2B).

Cloning and sequencing of the Tal-1 upstream region

The 369-bp cDNA sequence 5’ upstream from the Tal-1 methionine codon (NM_001107958) was determined, and was deposited in the DNA sequence database under accession no. AB728506. A comparison of rat Tal-1 cDNA sequence we determined (rat-TE) with that deposited in database (rat-DB) and the mouse Tal-1
cDNA sequence database (mouse) revealed an in-frame stop codon between two methionine codons (tga) in the rat-DB that was absent from the rat-TE. In the rat-TE, an in-frame stop codon (taa) was observed upstream of the 5' methionine codon (Fig. 3). The deduced amino acid sequences between the two methionine codons of mouse and rat-TE were found to share high homology (93.2%).

In situ hybridization and immunohistochemistry
Gene expression in the rat testis section was determined by ISH, and was found to be high in spermatocytes (Fig. 4). Immunohistochemistry revealed that TAL-1 protein expression occurred in the nuclei of the spermatocytes and spermatids, including the spermatagonia, according to the seminiferous epithelial cycle.

Expression was observed in the nuclei of the spermtids during stages VII and VIII (Fig. 5).

Discussion

CT antigens are powerful tools for cancer diagnosis and treatment. They are expressed in specific tumors, and normal organ expression is restricted to male germ cells, not adult somatic cells, even though the reason for this expression profile is not fully understood.7) We used DD to screen maturing rat testis-specific genes, focusing on novel CT antigen candidates related to T-ALL. Since T-ALL diagnosis and treatment of the younger generation is a high priority, T-ALL related CT antigens are urgently sought. Expression of TAL-1 was previously

Fig. 1. RT-PCR, Northern Blot, and Western Blot Analysis of Rat Tal-1 Transcript and TAL-1 Protein.
A, Tal-1 RT-PCR transcript analysis of 3-, 7-, 9-, and 15-week-old rat testis, and organs of 9-week-old rats (Cr, cerebrum; Lu, lung; He, heart; Lv, liver; Kd, kidney; Cl, colon; Bm, bone marrow; Sp, spleen; Th, thymus; Te, testis; Ov, ovary). Arrows indicate amplified 458-bp fragments. Rat Gapdh amplifications are indicated at the bottom. B, Tal-1 transcript analyzed by Northern blotting. Arrows on the left indicate positions of ribosomal RNA (28S rRNA and 18S rRNA). Arrows on the right indicate Tal-1 mRNA. Rat Gapdh expression is indicated at the bottom. C, TAL-1 protein analysis by Western blotting. Arrows on the left indicate positions of protein standards. Arrows on the right indicate TAL-1 signal position (34 kDa). The anti-GAPDH antibody signal is indicated at the bottom.

Fig. 2. NKX3.1, Gfi1, and VE-Cadherin Gene Expression in Maturing Rat Testis, and Tal-1 Expression in Human Organs.
A, NKX3.1, Gfi1, and VE-Cadherin RT-PCR transcript analysis of 3-, 7-, 9-, and 15-week-old rat testis, and organs of 9-week-old rats (Cr, cerebrum; Lu, Lung; He, heart; Lv, liver; Kd, kidney; Cl, colon; Bm, bone marrow; Sp, spleen; Th, thymus; Te, testis; Ov, ovary). Arrows indicate amplified fragments. Rat Gapdh amplifications are indicated at the bottom. B, Tal-1 RT-PCR transcript analysis of human fetal liver: Flv (63 spontaneously aborted male and female Caucasian fetus, ages 22–40 weeks), adult liver: Lv (51-year-old male Caucasian), bone marrow: Bm (56 Asian males and females, ages 22–95) and testis: Te (five Asians, ages 21–29). Human GAPDH amplifications are indicated at the bottom.
confirmed to be higher in the bone marrow cells of a pre-leukemia patient, and Tal-1 gene rearrangement has been observed in T-ALL patients. On the basis of this knowledge, we evaluated the possibility of using the Tal-1 gene as a CT antigen candidate.

TAL-1 has a bHLH domain and can be a positive regulator of several downstream genes expressed in the bone marrow in relation to the development of T-ALL. Tal-1 gene expression was strong in the rat testes after 7 weeks of sexual maturation. Stronger expression of Tal-1 in the bone marrow and in the testis, and faint expression in the lung, heart, and cerebrum in mature rats was detected, and it is known that faint expression of CT antigens occurs in many normal organs by sensitive RT-PCR analysis, even though the reason for the expression has not been documented. Tal-1 expression in the developing testis has not been reported to date, and we observed strong expression in humans as well as in rats. According to these results, we consider that this gene product can be classified as a novel CT antigen candidate, functionally related to stem-cell development. Rat Tal-1 mRNA approximated 28S rRNA size, in agreement with the size in the DNA database (4,297 bp). Western blotting detected a 34 kDa protein in the developing testis, bone marrow, and lung. Even the meaning of weak expression in lung is not understood. Protein signals in the kidney, colon, spleen, and thymus were larger than 34 kDa and can be considered non-specific. The rat Tal-1 mRNA sequence was deposited in the DNA sequence database under accession no. NM_001107958, in which the predicted 212 amino acid long protein is reported. Since the expected protein molecular weight was less than 34 kDa, we analyzed the upstream sequence of the proposed start methionine codon, considering the existence of the longer open reading frame (ORF). Rat upstream cDNA sequencing revealed no in-frame stop codons between the two methionine codons and the presence of an in-frame stop codon 5’ upstream of the methionine codon in Rat-TE. In this upstream region, the rat-TE DNA sequence was different from the Rat-DB in our experiment. As the sequenced region was present in one exon of the genome information and we amplified this area as a single band by PCR, we concluded that rat-TE is not a splicing variant of rat-DB DNA. Accordingly, the rat Tal-1 ORF was determined to be 329 amino acids long, and the TAL-1 molecular weight was predicted to be 34 kDa, in good agreement with our Western blotting results.

A novel finding of this study is the analysis of TAL-1 expression in the developing testis, which was widespread depending on the seminiferous epithelial cycle in the nucleus of the spermatogonia as well as the spermatocytes and spermatids. It is plausible that weak expression of Tal-1 was observed in the 3-week-old rat testis according to our results of Northern blotting, because at this stage pachytene spermatocytes appear. Our results of in situ hybridization indicate that the major Tal-1 expression was in the spermatocytes. It is remarkable that the translated TAL-1 protein in the spermatocytes was stably maintained in the spermatids. Since TAL-1 is expected to function as a transcription factor, the observed nuclear localization of the protein was plausible. Spz1 is another bHLH transcription factor, known to be specifically expressed in the testis, that plays a role in the MAPK signaling pathway. A novel finding of this study is the analysis of TAL-1 expression in the developing testis, which was widespread depending on the seminiferous epithelial cycle in the nucleus of the spermatogonia as well as the spermatocytes and spermatids. It is plausible that weak expression of Tal-1 was observed in the 3-week-old rat testis according to our results of Northern blotting, because at this stage pachytene spermatocytes appear. Our results of in situ hybridization indicate that the major Tal-1 expression was in the spermatocytes. It is remarkable that the translated TAL-1 protein in the spermatocytes was stably maintained in the spermatids. Since TAL-1 is expected to function as a transcription factor, the observed nuclear localization of the protein was plausible. Spz1 is another bHLH transcription factor, known to be specifically expressed in the testis, that plays a role in the MAPK signaling pathway. TAL-1 was expressed in the bone marrow as well as in the developing testis, and expression of it has been reported to be upregulated in pre-leukemic disorder, suggesting that it might be a transcription regulator of commonly activated genes among spermatogenic, bone marrow, and T-ALL cancer stem cells. Several TAL-1 target genes, including tumor suppressor NKX3.1, might participate in T-ALL development. NKX3.1 is a homeobox gene, that might be deeply involved in prostate development, and loss of its function is related to prostate carcinogenesis. The Tal-1 and NKX3.1 expression profiles in the testis were negatively correlated in our experiment. It may be hypothesized that Tal-1 downregulates gene expression of NKX3.1 in the maturing testis, leading to spermatogenic cell division and differentiation.

Future work ought to clarify gene expression regulation taking into account common mechanisms in stem cell development.
cell development and differentiation, leading to erythropoiesis, carcinogenesis, and spermatogenesis. These results should provide an understanding of the principal mechanism of TAL-1 in spermatogenesis, as well as identifying causes and promoting the treatment of T-ALL.

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