Tumor Cell-specific Transcription of a Murine Histocompatibility Class Ib Q5 Gene

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Abstract. Background: We previously reported that the Q5 gene product (Q5 antigen) was expressed on the surface of various tumor cells derived from H-2K (Qa-2- ) mice. The Q5 antigen has tumor-protective antigenicity in the syngeneic mice. Materials and Methods: Transcripts of the Qa region genes were analyzed by the RT-PCR method. Cell fractionation was performed with the MACS method and the phenotypes were estimated by flow cytometry analysis. Results: Transcripts of Q5 were produced by all tumor cell lines. The Q5 transcription was detected only in thymocytes and PBMCs of H-2K (AKR and C3H/He) mice. The phenotype of the PBMCs in which Q5 transcription takes place seems to be, at least partly, CD3-4-8- cells that might be related to CD3-4-8- spontaneous thymoma cells derived from an H-2K mouse. Conclusion: The expression of Q5 in tumor cells in general is a result of a change of transcriptional regulation associated with malignant transformation.

The mouse histocompatibility antigen genes are composed of classical (class Ia; H-2) genes and nonclassical (class Ib; Q and TL) genes. The class Ib genes are distributed in the Qa and Tla gene regions of chromosome 17. The Qa region is located adjacent to the class Ia gene region. While class Ia genes are highly polymorphic, class Ib genes are conservative. In the case of the C57BL/6 (H-2b) mouse, it has been reported that the Qa region contains 10 genes (Q1 to Q10, Q3 being a pseudogene) and the Tla region contains 29 genes (TL1 to TL29) (1). The products of both genes are consisted of glycosylated 3.7 to 4.1 kDa heavy chains that span the plasma membrane and a 1.2 kDa genetically conservative light chain known as β-2-microglobulin. Our knowledge is quite limited concerning regulation of the class Ib gene expression and the physiological roles of their products.

We previously reported that experimental tumor cells derived from Qa-2-negative H-2K mice generally express a cell surface molecule that is detected by some, but not all, Qa-2-specific monoclonal antibodies (mAbs) (2,3). The Qa-2 antigen is a normal alloantigen expressed on the surface of lymphocytes of certain strains of mice (4-6). The heavy chain of the Qa-2 antigen is a product of the Q7 or Q9 genes (7, 8), differing from each other by only one nucleotide. It has been reported, however, that in the Qa-2 region of H-2K mice (e.g. C3H/He, AKR etc.) the sub-region containing Q6 to Q9 genes are deleted, leaving only five Qa genes (Q1, Q2, Q4, Q5 and Q10) and a Q3 pseudogene (9, 10). Therefore, the antigen detected by some Qa-2-specific mAbs on the surface of tumor cells derived from H-2K mice, at least, cannot be the product of the Q7 or Q9 genes. We showed that the gene coding for the heavy chain of such an antigen molecule was the Q5 gene (2).

It was reported that the Q5 gene product (Q5 antigen) on the surface of tumor cells caused humoral immune reaction in the syngeneic tumor-bearing mice (3). Also, Q5-antigen-specific immunization caused protection of the mice against transplantation and metastasis of Q5 antigen-positive tumor cells (11). These results suggested that the mice were not tolerant to the Q5 antigen. Therefore the expression of the Q5 antigen, at least in adult mice, was tumor cell-specific.

On the other hand, it was reported that Q5 gene transcripts were detected by Northern hybridization and other methods in thymocytes and peripheral blood mononuclear cells (PBMCs) obtained from normal adult AKR mice (12, 13).

In the present study, we examined transcription of the Q5 gene by means of amplification by reverse transcription-polymerase chain reaction (RT-PCR) and determination of nucleotide sequences of the amplified products. We examined the Q5 transcription in each individual organ obtained from various mouse strains as well as in tumor cell lines established from mice of various strains. As a result,
transcripts of the Q5 gene were only detected in thymus cells and PBMCs obtained from H-2k mice. The transcripts were detected in all tumor cell lines tested irrespective of the H-2 haplotypes from which the cell lines were derived. The phenotypes of the AKR PBMCs in which Q5 transcription was detected, were further analyzed.

Materials and Methods

Animals, tumor cell lines and isolation of various organs. Male AKR (H-2k), BALB/c (H-2s), C57Bl/6 (H-2b) and C3H/He (H-2k) mice of 7 weeks of age were purchased from SLC Co., Ltd (Sizuoka, Japan). The mice were fed with a standard laboratory diet and water ad libitum until they were used at 10 to 12 weeks of age. Blood was collected by heart puncture under ether anesthesia using heparinized syringes. PBMCs were prepared from the blood using Lymphoprep™ system (Nycomed Pharma, Oslo, Norway). Various organs were excised from the mice after the bleeding. PBMCs and organs were also obtained from AKR mice bearing syngeneic BW5174 thymoma cells and C3H/He mice bearing syngeneic MH134 hepatoma cells, 7 days after subcutaneous inoculation of 2x10⁵ respective tumor cells to 10-weeks old mice. L929 fibrosarcoma and MH134 cell lines derived from a C3H/He mouse were generously provided by Dr. M. Yamazaki (Faculty of Pharmaceutical Sciences, Teikyo University, Kanagawa, Japan). MM2 mammary carcinoma and MH134 cell lines derived from a C3H/He mouse were maintained in vivo passages as ascitic cells in C3H/He mice. BW5174 cells were maintained in vitro. The W2K cell line is an SV40-transformed derivative of the C3H.2K kidney fibroblast cell line derived from a C3H/He mouse and shows malignant growth in vitro (14, 15). The cell line was a generously provided by the late Dr. N. Yamaguchi (Institute of Medical Science, the University of Tokyo, Japan). L929 and W2K cells were maintained in vitro using Dulbecco’s minimal essential medium (D-MEM) supplemented with 5% and 10% fetal bovine serum (FBS) respectively, and were passaged every 3 - 4 days, respectively. The MBT2 bladder carcinoma cell line derived from a C3H/He mouse, the BALB-MCE.12 mammary carcinoma cell line derived from BALB/c mouse, the WEHI-3 myelomonocytic leukemia cell line and the Y-1 adrenal tumor cell line were obtained from the Japan Cell Resources Bank. MBT2, BALB-MCE.12 and WEHI-3 were cultured in D-MEM supplemented with 10% FBS. Y-1 cells were cultured in Ham’s F10 medium supplemented with 10% FBS.

RNA isolation. PBMCs, freshly prepared as above, were washed with ice-cold saline containing 0.5mM EDTA. The following procedure has been described (16).

RT-PCR analysis. Synthesis of cDNA was carried out according to the manufacturer’s instructions as follows: 200 ng of the total RNA as the template, random hexamer or oligo dT12-18 as primers and SUPERSCRIPT™II RNase H- reverse transcriptase (Gibco BRL, Gaithersburg, USA) were mixed in a reaction mixture. The reaction mixture was subjected to PCR. The primers used to detect mouse glyceraldehydes-3-phosphate dehydrogenase (G3PDH) and five Q gene cDNAs (Q1, Q2, Q4, Q5 and Q10) are shown in Table I. They were synthesized by Amersham Biosciences K.K. (Tokyo, Japan). Amplification of cDNA was carried out using ExTaq™ (Takara-Bio, Shiga, Japan). After denaturation at 94°C for 1 min, 35 amplification cycles were performed. One cycle involved incubation at 60°C (G3PDH), 64°C (Q1, Q2, Q4 and Q10) or 66°C (Q5) for 1 min, at 72°C for 1 min and 94°C for 30 sec.

Cloning and sequencing of PCR-amplified DNA. The procedures have been described (16).

Cell fractionation. The T cell fraction was prepared from PBMCs or thymus cells obtained from normal AKR or C3H/He mice using the Pan T Cell Isolation Kit (Miltenyi Biotec GmbH, Gladbach, Germany), which contained beads to which anti-CD45, anti-CD49b, anti-CD11b and anti-Ter-119 mAbs were conjugated. T cell fraction and non-T cell fraction were isolated according to the manufacturer’s instructions. CD4⁺ cells and CD8⁺ cells were recovered from AKR PBMCs using magnetic beads conjugated with anti-mouse CD4 (LST4) or anti-mouse CD8a (Ly-2) mAb (MACS, Miltenyi Biotec GmbH, Gladbach, Germany).

Flow cytometry analysis. Detection of the expression of lymphocyte surface marker molecules was performed using a flow cytometer (Epics XL, System II, Beckman-Coulter Inc., California, USA). The following mAbs were used: FITC-conjugated anti-mouse CD3-ε mAb (hamster IgG, group 1, 1, BD Biosciences, California, USA), FITC-conjugated anti-TNP mAb (hamster IgG, group 1, 1, BD Biosciences, California, USA) as an isotype-matched control, FITC-conjugated anti-mouse CD4 mAb (rat IgG2b, Beckman-Coulter Inc., California, USA), FITC-conjugated rat IgG2b (LONDP-57, Beckman-Coulter Inc., California, USA) and PE-conjugated rat IgG2a (LONDP-16, Beckman-Coulter Inc., California, USA) as an isotype matched control.

Results

Transcription of the Q5 gene in tumor cell lines. First, we examined the expression of the Q1, Q2, Q4, Q5 and Q10 genes in various murine malignant cell lines to show that transcription of the Q5 gene generally takes place. We performed RT-PCR analysis using RNA samples extracted from each cell line as the templates and specific primer sets designed on the basis of reported DNA sequences of the genes of C3H/He (Table I). The tumor cells used contained seven H-2k, one H-2b and one H-2d cell lines. They also contained those maintained by in vitro culture. As shown in Figure 1-A, transcription of the Q5 gene was detected in various tumor cells except Y-1. The cells in which Q5 transcripts were detected contained those with H-2 haplotypes ‘k’ and ‘d’. Amino acid sequences of the Q5 molecules are well conserved among various strains of the mouse, but the nucleotide sequences of the Q5 genes have some heterogeneity according to the H-2 haplotypes. Although we could not trace the origin of the Y-1 cells in the literature, the Q5 transcripts were detected in Y-1 cells using primers designed to amplify Q5 transcripts in the H-2b mouse (Figure 1-B). The Q5 transcription was confirmed by
sequencing the amplified DNA in all cases. The resulting sequences matched those of the reported sequences of mice of the respective H-2 haplotype (ACCESSION: X16423, GeneBank). Therefore transcription of the Q5 genes was demonstrated in all malignant cell lines tested. On the other hand, transcription of Q1, Q2, Q4 and Q10 genes was negligible in all cell lines tested.

Detection of transcription of the Q5 gene in various organs and cells from normal adult and tumor-bearing mice. To study whether expression of the Q5 gene is under transcriptional regulation, total RNA samples prepared from various organs isolated from various normal adult mice were analyzed by RT-PCR. The mice included AKR, C3H/He, BALB/c, C57BL/6 and AKR bearing syngeneic BW5147 thymoma cells and C3H/He bearing syngeneic MH134 hepatoma cells. When tumor-bearing mice were used, the tumor cells were transplanted subcutaneously and the mice were sacrificed when the tumor reached 5 to 7 mm in diameter. The presence of Q5 transcripts was examined in the RNA samples obtained from H-2k, H-2d and H-2b mice using primers designed to detect the Q5 sequences in H-2k, H-2d and H-2b cells, respectively. The results are shown in Figure 2. Q5 transcripts were detected only in RNA samples derived from PBMCs and thymus of AKR and C3H/He mice (both H-2k). By sequencing the amplified DNA, transcription of the Q5 gene was again confirmed. Apart from these cells, Q5 transcripts were not detected in any of the organs tested. From these findings, it is evident that transcription of the Q5 gene is restricted to tumor cells, thymus cells and PBMCs. Therefore, the expression of the Q5 antigen on the surface of various tumor cells is a result of changes in the transcriptional regulation. The Q5 transcript became undetectable in PBMCs and thymus of the tumor-bearing C3H/He and also in thymus of the tumor-bearing AKR (Figure 3). These results suggest that the presence of tumor cells somehow prevents the Q5 transcription in these cells.

Phenotypes of AKR PBMCs with Q5 transcription. As shown above, Q5 transcripts were detected generally and almost exclusively in tumor cells. The only exceptions among normal adult organs and cells were thymus and PBMCs of AKR and C3H/He mice. It is known that AKR mice acquire spontaneous thymoma with aging (17-20) and show age-related expression of the histocompatibility class I b thymus-leukemia (TL) antigen (21). Although the AKR mice that we used did not have the disease yet, we speculated that the Q5 transcription in AKR thymus represented possible pre-thymoma cells.

Table I. Primers for PCR.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Antisense Primer (5’ to 3’)</th>
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<tr>
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</table>

Figure 1. Detection of transcription of Q genes in various tumor cell lines (A) and detection of transcription of Q5 gene in the Y-1 cell line (B) analyzed using RT-PCR. A: Primers specific to Q1k, Q2k, Q4k, Q5k and Q10k were used. The analyzed cell lines are shown at the top of the figure. B: Primers specific to Q5b were used. ’M’ means molecular weight-marker. Primers specific to G3PDH were used to detect transcription of the enzyme as an internal control. RT-PCR amplified cDNAs were visualized by ethidium bromide staining after agarose-gel electrophoresis.
contained in the sample and that the transcript detected in AKR PBMCs resulted from the pre-thymoma cells released into the blood. We tried to clarify the phenotypes of the AKR PBMCs in which Q5 transcripts were detected and compare them to those of the AKR-derived BW5147 thymoma cell line. The AKR PBMCs were obtained and T cells were negatively selected by depletion of CD45R+, CD49b+, CD11b+ and Ter-119+ cells. As shown in Figure 4-A, B, 96.4% of the cells in

<table>
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<tr>
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Figure 2. Detection of transcription of Q5 genes by RT-PCR in various organs and cells obtained from a variety of mouse strains. For the detection in organs from AKR (A), BALB/c (B), C57BL/6 (C) and C3H/He (D), primers specific to Q5, Q5k, Q5b and Q5k were used, respectively. Organs and cells are shown at the top of the figure. The BW5147, MC.E12 (BALB-MC.E12) and Y-1 tumor cell lines were used as positive controls. Primers specific to G3PDH were used to detect transcription of the enzyme also as a positive control.
the T cell fraction were CD3⁺ and 96.1% of the cells in the non-T cell fraction were CD3⁻. The resulting cells were subjected to detection of Q5 transcripts by RT-PCR. The transcripts were detected almost to the same extent in both T and non-T cell fractions (Figure 4-E). On the other hand, when thymocytes were fractionated into T and non-T cell fractions using the same method, both of them contained CD3 strongly-positive cells, CD3 weakly-positive cells and CD3 negative cells (Figure 4-C, D). In this case, the Q5 transcripts were detected only in the non-T cell fraction (Figure 4-E). CD4⁺ cells and CD8⁺ cells were also isolated from the PBMCs using magnetic beads conjugated with CD4-specific mAb or CD8-specific mAb, respectively. As shown in Figure 5-A, Q5 transcripts were not detected in either CD4⁺ (98.8% by FCM analysis, data not shown) cells or CD8⁺ (99.7% by FCM analysis, data not shown) cells. These results suggest that Q5 transcription may not be related to T cell markers. In a parallel experiment, it was shown that BW5147, an AKR-derived thymoma cell line, was CD3⁻, CD4⁻ and CD8⁻ (Figure 5-B). Presumably Q5-transcribing cells are not homogeneous and there are CD3⁻4⁻8⁻ pre-thymoma cells in the AKR thymus cells and PBMCs, which are related to the CD3⁻4⁻8⁻ thymoma cells. Further characterization of the cells was not successful at this stage due to the low quantity of cells in question and lack of an appropriate mAb specific to the Q5 antigen.

We examined transcription of the Q5 gene in various organs and cells in another H-2k mouse, C3H/He, which do not produce spontaneous thymoma. Contrary to our expectations, Q5 transcripts were detected in thymus and PBMCs of C3H/He. This result does not support the assumption that the Q5-transcribing cells are pre-thymoma cells and suggest that the phenomenon is more or less related to H-2k haplotype of the mice. Q5 gene transcription in thymus and PBMC, as well as various organs of tumor-bearing AKR or C3H/He 7 days after subcutaneous transplantation of syngeneic tumor, were also examined. It was found that the transcripts were not detected in thymus of the AKR, nor in thymus and PBMCs of the C3H/He. The significance of such regulation of Q5 transcription is not clear at this moment.
Discussion

In a previous report, we described that Q5 transcripts were detected by RT-PCR in the AKR-derived thymoma cell line BW5147, but not in AKR thymus (2). This result differed from the present results with regard to AKR thymus. The discrepancy may be attributed to the difference of affinity of the primers used to Q5 cDNA. It seems that the amount of the Q5 transcript is so low in these cells that the transcript can be detected only when high affinity primers were used, while that in BW5417 was sufficiently high to be detected even when using low affinity primers. There have been reports which show transcription of Q5 in AKR mice. Schwemmel et al. (12) reported that Q5 transcripts were detected in AKR tumor cells, AKR embryo and thymus of young AKR. Reyes-Engel et al. (13) found Q5 transcripts in AKR tumor cells, thymus of 1- to 2-week-old AKR mice and testis of 5- to 7-week-old AKR mice. These results were obtained by Northern blotting or by RNase protection assay. Since there are large number of class I genes which

Figure 4. Flow cytometry analysis of T and non-T cell fractions obtained from AKR PBMCs and thymus cells (A, B, C and D) and detection of transcription of Q5 genes by RT-PCR in the T and non-T cell fractions (E). The cell fractionation was carried out using the Pan T Cell Isolation Kit as described in Materials and Methods. The resulting cells were stained by anti-CD3 mAb and were subjected to analysis by flow cytometry. A: T cell fraction from PBMCs, B: Non-T cell fraction from PBMCs, C: T cell fraction from thymus cells, D: Non-T cell fraction from thymus cells. E: PCR product of T cell fraction from PBMCs (lane 1), non-T cell fraction from PBMCs (lane 2), T cell fraction from thymus cells (lane 3) and non-T cell fraction from thymus cells (lane 4) were applied. Primers specific to Q5 were used. Primers specific to G3PDH were used to detect transcription of the enzyme also as a positive control.
are highly homologous to each other, there is still a possibility that their findings resulted from cross-reactivity of the probes used to other class I cDNAs. Because of this ambiguity, we examined transcription in more detail and used a newly designed set of primers, obtaining the results shown above. Also, we confirmed transcription of the Q5 gene by sequencing the RT-PCR-amplified cDNA. Our results proved that transcription of the Q5 gene was not strictly tumor cell-specific. On the other hand, we proved that the Q5 gene product expressed on the surface of tumor cells was immunogenic to the syngeneic hosts. It can be speculated that in thymus and PBMCs there are some post-transcriptional regulations that inhibit the expression of the Q5 antigen molecule on the surface of these normal cells. However, it is not feasible at present to clarify such a hypothesis because of difficulty in obtaining mAbs with exact specificity to the Q5 antigen. The transcription of the Q5 gene in adult thymus and PBMCs seemed to be down-regulated in tumor-bearing mice. The mechanism of this phenomenon is not known, although some malignancy-associated changes of cytokines in the environment of the cells may affect the transcription.

The transcription of the Q5 gene, not only in AKR thymus and PBMCs but also in C3H/He thymus and PBMCs, may show that it is related to the H-2^k haplotype of the mice rather than to the possible pre-thymoma cells in the cell preparation, as we speculated above. There is still a possibility, however, that C3H/He thymus and PBMCs also contain pre-thymoma cells and that their development to cancer is suppressed by some reason. If this were the case, it could be said that transcription of the Q5 gene took place specifically either in tumor cells or in pre-tumorous cells.

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References


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