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Experimental model of micronodular thymic neoplasm with lymphoid stroma

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Abstract

Background: Micronodular thymic neoplasm with lymphoid stroma (MNT), a subtype of thymic tumor, is histopathologically characterized by micronodular thymic epithelial cell nests with lymphoid stroma. Despite the distinct histopathology of MNT, its pathogenesis remains unclear.

Methods: In this study, we aimed to examine a thymic tumor harboring thymic epithelial and lymphoid cells in a nonobese diabetic/severe combined immunodeficiency mouse.

Results: The excised tumor cells were cultured in vitro and comprised epithelial tumor cells and lymphoid cells. During a three-dimensional cell culture, the epithelial tumor cells formed micronodular cell nests surrounded by lymphoid stroma. Notably, the lymphoid cells underwent apoptosis when they were separated from the epithelial tumor cells. Cutaneous transplantation of the cultured epithelial cells with splenocytes from BALB/c mice led to tumor formation, and these cells demonstrated a histopathology similar to that of human MNT in a nonobese diabetic/severe combined immunodeficiency mouse.

Conclusion: Given its overlapping features with human MNT, the transplanted tumor could serve as an experimental model of this disease.

KEYWORDS
lymphoid stroma, micronodular thymic neoplasm with lymphoid stroma, three-dimensional cell culture, thymic epithelial cell

INTRODUCTION

Micronodular thymic neoplasm with lymphoid stroma (MNT) is a rare subtype of thymic neoplasm that is histopathologically characterized by micronodular thymic epithelial cell nests with lymphoid stroma. MNT was first reported by Suster and Moran,[1] and since then accumulated case reports have indicated that this tumor represents a spectrum of micronodular thymic epithelial tumor cell proliferations, varying from micronodular thymoma to thymic carcinoma with lymphoid stroma and with intratumorous lymphoma.[2–6]

The biological properties of thymic epithelial cells, including their ability to attract and harbor lymphocytes to promote lymphocyte development,[7] might be related to the lymphoid stroma formation of micronodular thymic neoplasm. However, the pathobiological and molecular mechanisms (e.g., humoral factor pathway) underlying abundant lymphoid stroma formation in micronodular thymic neoplasm remain to be elucidated.

In this study, we report a putative experimental model of MNT, which might be useful to elucidate the pathobiological property of MNT.

METHODS

Mouse, cells, and cell culture

Incidental thymic tumor was excised from a nonobese diabetic/severe combined immunodeficiency (NOD/SCID; NOD.CB17-*Prkdc*^{scid}/J) mouse (Charles River Laboratories). Dulbecco's modified Eagle's medium (DMEM) has been

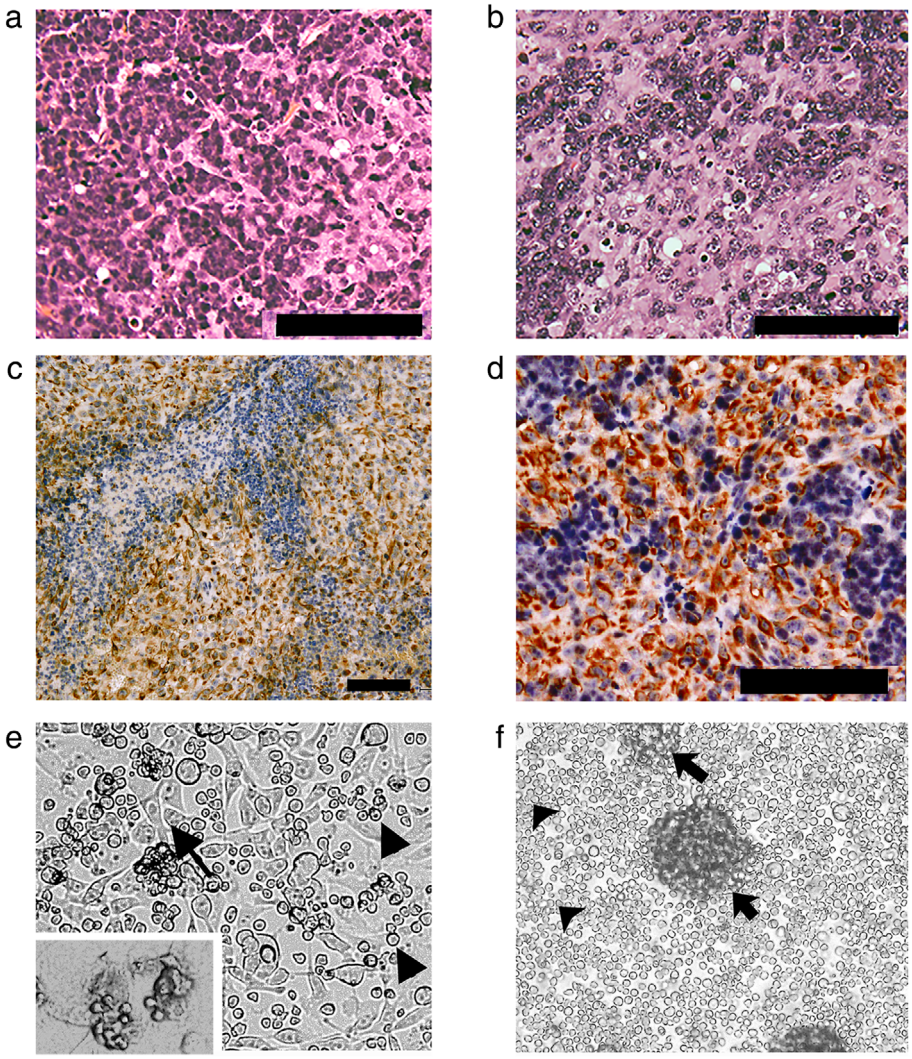

FIGURE 1 Morphological features of the thymic tumor. (a) and (b) Tumor composed of variably sized epithelial cell nests surrounded by lymphoid cells in a hematoxylin and eosin-stained tissue section. (c) and (d) Epithelial cells exhibited pan-cytokeratin immunoreactivity by immunohistochemical staining. Scale bar represents 100 μm. (e) and (f) Epithelial and lymphoid cells, indicated by arrows and arrowheads, respectively, was also found in standard dishes (e) and three-dimensional culture (f). Note the grouped lymphoid cells which were harbored by epithelial cells (e, insert).

used as a culture medium for various thymic cells, including murine thymic organ cultures.[8] Therefore the resected tumor was minced for cell culture in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS) without any antibiotics. The tumor was formalin-fixed, paraffin-embedded, and sectioned for histopathological analysis.

For a three-dimensional cell culture, the cells were cultured in DMEM containing 10% FBS on a six-well plate (1×10^6/well) coated with ultra-low attachment surface, which prevents cell attachment (Corning Life Sciences).

Immunofluorescent cytochemical staining

Immunofluorescent cytochemical staining was performed as previously described.[9,10] Rat antimouse CD3 (clone 17A2) conjugated with fluorescein isothiocyanate (FITC) (catalog no. 1101015), rat anti-mouse CD4 (clone GK1.5) conjugated with phycoerythrin (PE) (catalog no. 1102040), and rat anti-mouse CD8 (clone 53–6.7) conjugated with FITC (catalog no. 1103525) were purchased from Fujifilm Wako Pure Chemical Co. In brief, the cells were incubated with or without 1:100 diluted antibodies at 4°C for 1 h. After washing with phosphate-buffered saline (PBS), the cells were analyzed with a Guava EasyCyte cell analyzer (Hayward).

For the detection of cytoplasmic CD3, the cells were fixed with acetone on a slide glass, permeabilized with 0.1% TritonX-100, and then blocked with 10% goat serum. They were then incubated with or without 1:100 diluted hamster antimouse CD3 conjugated with Alexa Fluor 488 (MCA2690A488) (Bio-Rad) at room temperature for 30 min and washed with PBS. The cells were imaged under a confocal laser scanning microscope (Leica TCS SP8).

Detection of apoptosis

The number of cells undergoing apoptosis was quantified with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) (PromoCell GmbH). In brief, 1×10^4 cells under different culture conditions were harvested,

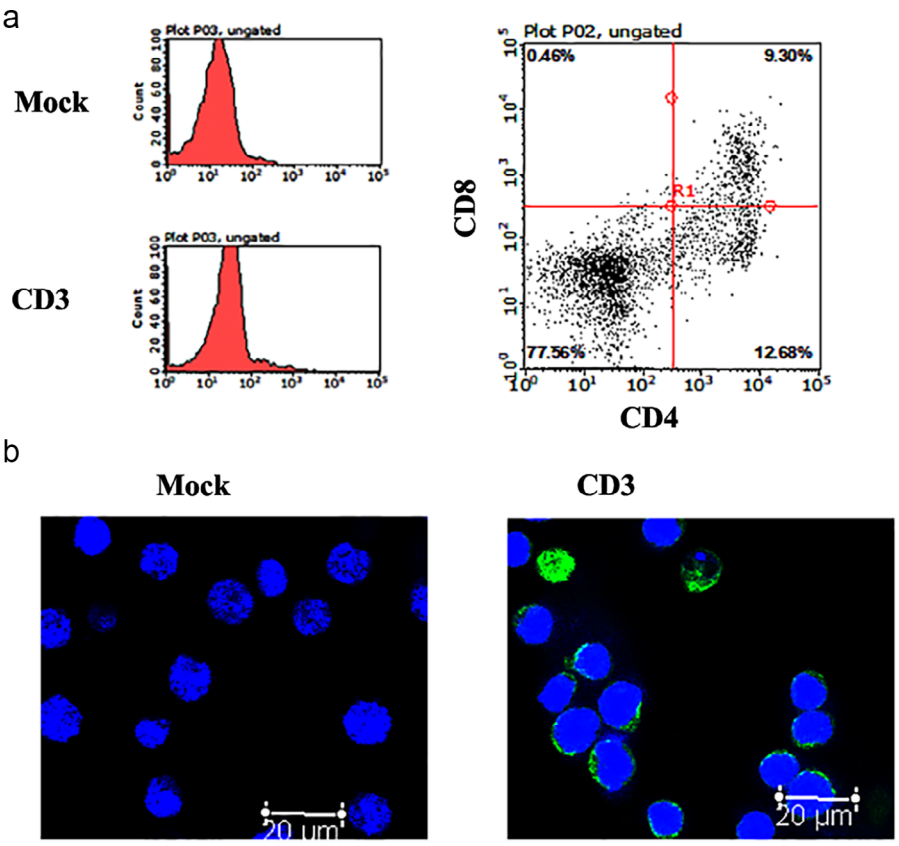

FIGURE 2 Representative immunofluorescent staining of cultured lymphoid cells. (a) Lymphoid cells exhibited dull staining on the cell surface with anti-CD3. Major lymphoid cells did not exhibit immunoreactivity with anti-CD4 or anti-CD8 antibody. Approximately 20% of lymphoid cells demonstrated CD4 immunoreactivity, while approximately 10% lymphoid cells showed CD8 immunoreactivity. (b) Fixed lymphoid cells were stained without or with anti-CD3 antibody conjugated with Alexa Fluor 488 (right or left, respectively). Note the green CD3 immunostaining in the cytoplasm of lymphoid cells. DAPI (4′, 6-diamidino-2-phenylindole) blue staining was also used to visualize the nuclei.

washed, resuspended in binding buffer, mixed with annexin V-FITC and PI, and then analyzed as previously described.[11]

Cellular fragmented DNA was extracted using Apoladder Ex (Takara Bio) in accordance with the manufacturer's instructions. The DNA was resuspended and electrophoresed in a 2% agarose gel at 50 V for 2 h. DNA was visualized using GelRed nucleic acid gel stain (Biotium, Inc.) and photographed under UV light using an Invitrogen iBright 1500 gel imaging system (Thermo Fisher Scientific).

Transplantation

BALB/c mice were purchased from Charles River Laboratories, Japan. Three-dimensional cell cultured cells were plated on to standard 35 mm tissue culture dishes (BD Falcon) for 48 h. Epithelial cells attached to the dish were separated from floating lymphoid cells and expanded with DMEM supplemented with 10% FBS. Then, 3.4×10^7 epithelial cells were again cultured in ultra-low attachment surface plates for 16 h. The harvested epithelial cell spheroids and 3.0×10^7 BALB/c splenocytes were mixed in VitroGel hydroGel matrix (TheWell Bioscience Inc.), thereafter cutaneously injected into the soft tissue of the thighs of 10-week-old NOD/SCID mouse. Two weeks after transplantation, the tumor was excised, formalin-fixed, paraffin-embedded, and then sectioned for histopathological analysis.

Immunohistochemical staining

The tissues were immunostained with anti-pan-cytokeratin antibody, clone AE1/AE3 (Leica Biosystems) using a Mouse to Mouse HRP Kit (cat no. MTM001, ScyTek Laboratories) according to the manufacturer's instructions. The tissues were also immunostained with rat monoclonal antibody to CD45R/B220 (clone RA3-6B2, Proteintech) using Nichirei -Histofine Simple Stain kit (cat no. 414311F, Nichirei Biosciences Inc.).

RESULTS

Thymic tumor of an NOD/SCID mouse

NOD/SCID mice were purchased for the xenoplantation assays in another experiment.[12] For this assay, we acquired 10 NOD/SCID mice. A 14-week-old NOD/SCID mouse exhibited decreased mobility and food consumption before the tumor xenoplantation experiment. Surprisingly, this mouse had a thymic tumor, approximately 1.0×0.5 cm in size. The other nine NOD/SCID mice were healthy and were used for xenoplantation experiments. The autopsies revealed no thymic tumors in these mice. Histological examination revealed that the tumor exhibited multiple polygonal cell nests with lymphoid stroma (Figure. 1a, b), a morphological feature similar to that of a human multinodular thymic tumor with a lymphoid stroma. To confirm that these

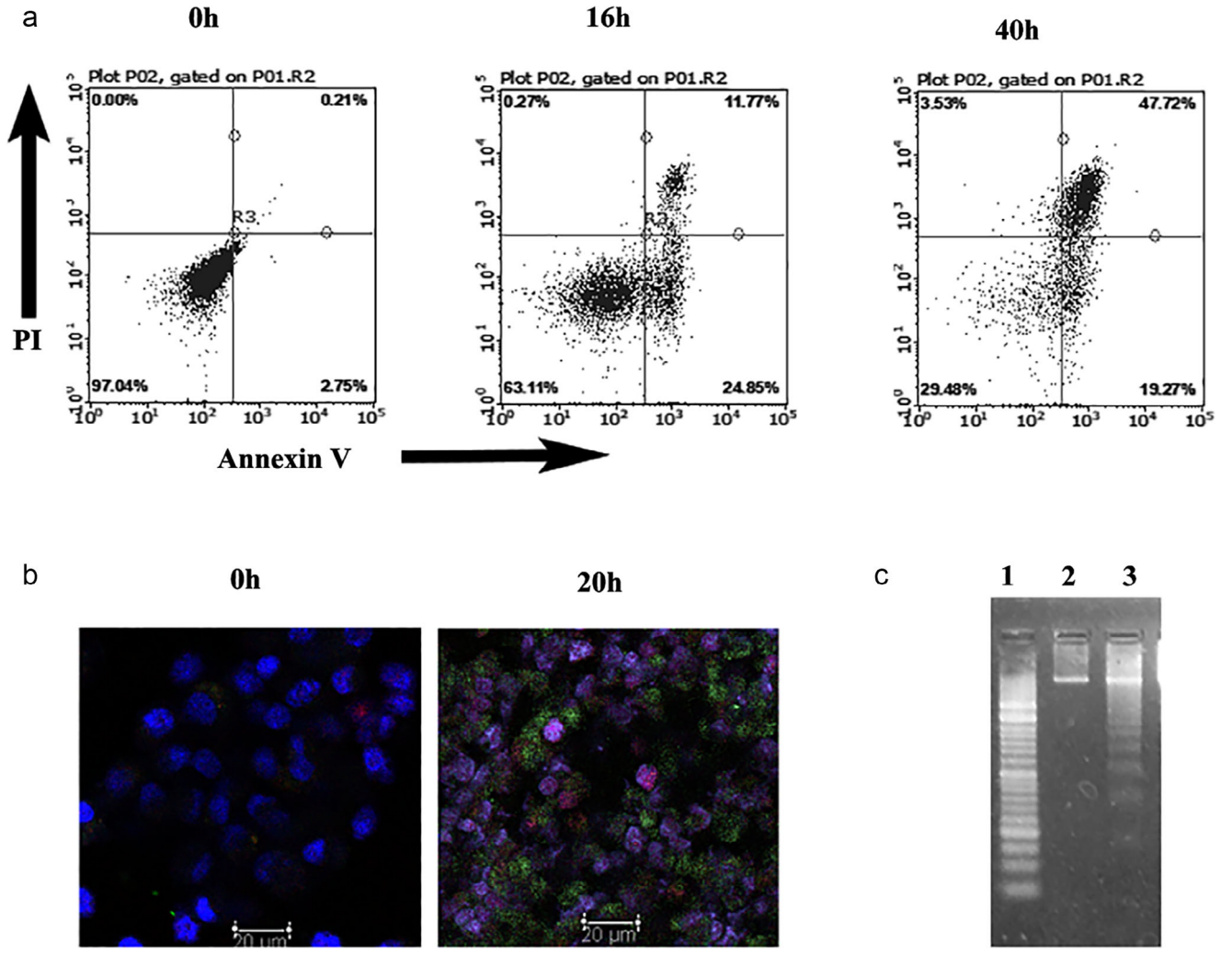

FIGURE 3 Lymphoid cells are dependent on epithelial cells in vitro. (a) Floating lymphoid cells were separated from epithelial cells by aspiration and then cultured for 16 and 40 h. The number of cells undergoing apoptosis was quantified with a FITC-conjugated annexin V and PI assay. Note the populations of cells: viable and not undergoing apoptosis (annexin V-FITC and PI negative); undergoing apoptosis (annexin V-FITC positive and PI negative); and end-stage apoptosis or already dead (annexin V-FITC and PI positive). (b) Annexin V-FITC and PI staining of lymphoid cells analyzed by confocal fluorescence microscopy. Blue indicates nuclear staining by DAPI. Scale bar represents 20 μm. (c) DNA fragmentation assay in agarose gel. Note the apoptosis ladder in the lymphoid cells separated from epithelial cells (lane 3) but not in the coculture with epithelial cells (lane 2). 50 bp DNA ladder molecular marker is shown in lane 1.

polygonal cells had an epithelial cell phenotype, immunohistochemical staining was performed. Notably, multiple epithelial cell nests exhibited a pan-cytokeratin, AE1/AE3 immunoreactivity and therefore appeared to have an epithelial cell phenotype (Figure 1c, d).

Cell culture of the thymic tumor

We cultured thymic tumors for further characterization. The organization of tumor cells in the three-dimensional culture elucidates the pathobiological properties of the tumor, especially its microenvironment.[13] Therefore, we developed a standard two- and three-dimensional cell culture. Excised tumor cells were composed of two cell mixtures, namely, dish-attached epithelial cells and floating lymphoid cells in standard 35 mm dishes (Figure 1e). Two weeks after cell culture in standard dishes, the tumor cells were transferred to a three-dimensional cell culture. As demonstrated in Figure 1f, the tumor cells again formed two cell patterns, namely, epithelial spheroid cell nests and surrounding lymphoid cells. This indicates that epithelial tumor cells form tumor nests support the lymphoid stroma in the present tumor. This three-dimensional cell culture might provide a useful platform for identifying the biological characteristics of micronodular thymic neoplasms with lymphoid stroma.

Immunofluorescence cytochemical staining

Subsequently, we examined the lymphoid stroma phenotype. Cytoplasmic CD3 expression was evaluated in acetone-fixed and TritonX-100 permeabilized lymphoid cells by

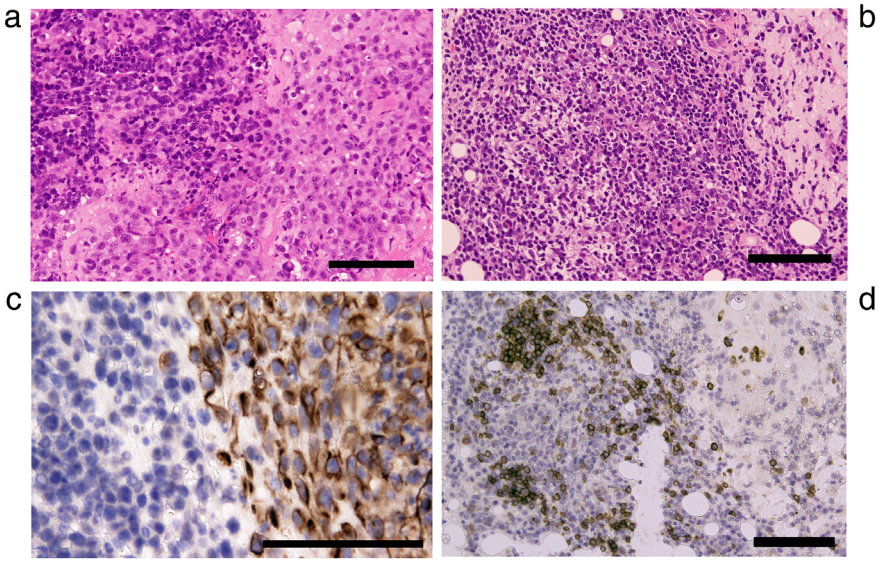

FIGURE 4 Histopathological examination of transplanted tumor. (a) and (b) Transplantation of epithelial cells with splenocytes resulted in tumor formation. Histopathological examination revealed that the tumor comprised multiple nodular epithelial cell nests and abundant lymphoid stroma (hematoxylin and eosin stain). (a) Represents a region with mixture of lymphoid stroma (upper left) and epithelial cell nest (lower right). Lymphoid follicle was observed in lymphoid stroma (b). (c) and (d) Immunohistochemical staining demonstrated that epithelial cells expressed pan-cytokeratin immunoreactivity (c), while lymphoid cells exhibited B cell phenotype (d). Scale bar represents 100 μm.

confocal microscopy, whereas surface CD3, CD4, and CD8 expression was examined in intact lymphoid cells, using a Guava EasyCyte cell analyzer. Immunofluorescent cytochemical staining demonstrated that the lymphoid cells of the present thymic tumor exhibited surface $CD3^+CD4^-CD8^-$, $CD3^+CD4^+CD8^+$, or $CD3^+CD4^+CD8^-$ and cytoplasmic $CD3^+$ phenotype (Figure 2). This indicated that the present tumor harbored a mixture of T cells at various levels of intrathymic differentiation: CD4 and CD8 double-negative and double-positive T cells and CD4 single-positive T cells.

Lymphoid cells separated from epithelial tumor cells undergo apoptosis

During the three-dimensional cell culture, the separation of floating lymphoid cells from epithelial cells decreased the proliferation of lymphoid cells, while the separation of lymphoid cells did not alter the growth of epithelial cell nests (Figure S1). Subsequently, we determined whether or not the proliferation of lymphoid cells is dependent on epithelial cells. Notably, the lymphoid cells underwent apoptosis when they were separated from the epithelial cells (Figure 3). This suggests that epithelial tumor cells might support stromal lymphocyte growth in thymic tumors.

Transplantation of epithelial cells with splenocyte formed a tumor that is histopathologically similar to MNT

Finally, we contemplated whether epithelial tumor cells also supported B cell growth. We xenoplanted splenic lymphocytes, composed of B cells with epithelial tumor cells. As shown in Figure 4, the transplanted tumor exhibited micronodular thymic neoplasm with abundant lymphoid stroma. Immunohistochemical staining demonstrated that the lymphoid stroma was composed of B cells with lymphoid follicles. The transplanted tumor was histopathologically similar to human MNT.

DISCUSSION

Aged NOD/SCID mouse is highly affected by thymic pre-T cell lymphoblastic lymphoma. Prochazka et al. found thymic lymphoma in 24 of thirty-six 40-week-old NOD/SCID mice.[14] Previous xenoplant experimental assays[12] revealed the formation of thymic lymphoma in aged NOD/SCID mice. However, the present thymic tumor was found in a relatively young 14-week-old mouse.

Histopathological examination revealed that the tumor exhibited micronodular thymic epithelial cells around abundant lymphoid cells. Immunostaining revealed that the tumor comprised cytokeratin-positive thymic epithelial cells and $CD3^+$ lymphoid cells. AE1/AE3 cytokeratin immunoreactivity was used to highlight the thymic epithelial cells.[15] Immunofluorescence staining also revealed that tumor $CD3^+$ T cells were at various intrathymic differentiation stages: CD4 and CD8 double-negative and double-positive T cells and CD4 single-positive T cells. Three-dimensional cell culture demonstrated the autonomous growth of epithelial tumor cells, which were highlighted as micronodular cell nests. Consequently, we believed that the tumor was composed of micronodular thymic epithelial nests with intrathymic lymphocyte stroma.

Notably, the lymphoid cells underwent apoptosis when separated from the epithelial cells. In contrast, the epithelial cells grew, regardless of the presence of lymphoid cells in their surroundings. This indicates that epithelial cells play a central role in thymic tumors. Indeed, transplantation of epithelial cells with nontumorous splenocytes formed a tumor with CD45R/B220-positive B-cell lymphocyte stroma, with a morphology similar to that of human MNT.

MNT with lymphoid stroma was originally described by Suster and Moran[1] as a characteristic of multiple micronodular thymoma epithelial cell nests with abundant B cell stroma, often with lymphoid follicles. Interestingly, several variations have been added to the concept of micronodular thymic neoplasm with lymphoid stroma; epithelial components varied from thymoma to thymic carcinoma, and lymphoid stroma was also associated with T cells.[2–6] The thymic tumor was composed of AE1/AE3 anticytokeratin cocktail positive epithelial cells, which showed autonomous growth, whereas the lymphoid stroma consisted of T cells, which declined in number, due to apoptosis, as a consequence of absent epithelial cell nests. This study demonstrated that T cell stroma could be replaced by CD45R/B220-positive B cells. This supports the idea that thymic epithelial tumor cells play a crucial role in the tumorigenesis of micronodular thymic neoplasms with lymphoid stroma, similar to other thymomas or thymic carcinomas. The murine tumor model may support the idea that micronodular thymic tumors with lymphoid stroma, unlike thymic lymphoepithelial tumors or gastric carcinomas with lymphoid stroma, are not directly related to Epstein–Barr virus infections,[2,6] which are endemic to humans.[16]

In conclusion, the transplanted tumor serves as an experimental model for human MNT. Further examination is underway to clarify whether epithelial tumor cells sustain lymphoid stroma via the humoral factor pathway and/or direct cell-to-cell interaction.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ETHICS STATEMENT

The experimental protocol was approved by the Animal Care Committee of Gifu Graduate School of Gifu, Japan (Approval no. 2022–123).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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