Adoptive Transfer of tsk Skin Fibrosis to +/+ Recipients by tsk Bone Marrow and Spleen Cells (42979)

MELINDA A. WALKER,* RUSSELL A. HARLEY,† FRANK A. DELUSTRO,‡ AND E. CARWILE LEROY*

Department of Medicine, Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29425; Department of Pathology,† Medical University of South Carolina, Charleston, South Carolina 29425; and Connective Tissue Research Laboratories,‡ Collagen Corporation, Palo Alto, California 94303*

Abstract. **The tight skin mouse (tsk/+) is an autosomal dominant example of inherited fibrosis whose pathogenesis is unclear. Autoimmune phenomena have been described previously. This study demonstrates the adoptive transfer of skin fibrosis to lethally irradiated syngeneic +/+ recipients by the transplantation of both bone marrow and spleen cells. The recipient skin fibrosis was not associated with mast cell proliferation or degranulation as it is in the tsk/+ mouse, representing for the first time a dissociation between mast cells and fibrosis and introducing the potential for direct T cell-fibroblast interactions.** [P.S.E.B.M. 1989, Vol 192]

In 1976, Green *et al.* (1) described an autosomally dominant mouse mutation called tight skin (tsk/+) which is located on chromosome 2. Homozygous (tsk/tsk) mice die *in utero*; heterozygous (tsk/+) mice have tight skin due to an exuberant increase of the subcutaneous connective tissue, as well as other changes of the lungs, cartilage, and bone. Menton and Hess (2) characterized the connective tissue and fiber organization of the tsk/+ mouse and compared the cutaneous abnormalities with those of scleroderma in man. In addition, Szapiel *et al.* (3) described a pulmonary lesion with histologic features of emphysema.

DeLustro *et al.* (4) observed a significant delayed-type hypersensitivity response to elastase-solubilized lung peptides in tsk/+ mice unaccompanied by significant immune responses to types I or IV collagens. They also demonstrated that adoptive transfer of spleen cells resulted in acquired delayed-type hypersensitivity responsiveness which was abrogated by prior treatment with anti-thy-1.2 serum and complement, indicating that tsk/+ mice generate a cell-mediated autoimmune response to elastin peptides or to cross-reacting epitopes. They did not detect humoral antibody to the antigens studied in either tsk/+ or +/+ mice at any age. Skin fibrosis in the early adoptive transfer experiments was not studied.

We have previously reported (5) an increased number and enhanced degree of degranulation of dermal mast cells in tsk/+ mice; also, we quantified the subcutaneous fibrous layer that increases in width with age. After oral treatment of tsk/+ mice with disodium cromoglycate, which inhibits mast cell degranulation, we observed a decreased degranulation of dermal mast cells and a pronounced decrease in the subcutaneous fibrous layer (6). These findings suggested that the mast cell may play a role in the skin fibrosis of the tsk/+ mouse. Other interpretations involving pharmacologic effects on cells other than mast cells (fibroblasts, T cells) were not studied.

Increased numbers of mast cells have been reported in scleroderma by Hawkins *et al.* (7). Skin lesions resembling scleroderma have been observed in chronic graft-versus-host disease (8-11) and mast cell dysfunction has been noted in a mouse model of graft-versus-host disease (12, 13). There is evidence that mucosal mast cells may be both under T cell control and also may modulate the responses of T cells and of other immune cells (14–18). The mechanisms of connective tissue mast cell regulation are unknown. Mast cell differentiation appears to be regulated by factors from $Ly1^+2^-Ia^-$ T cells (18). In addition, functional interrelationships have been noted between mast cells and both natural killer and suppressor T cells (19–21).

We wanted to ask if the mast cell is essential in the skin fibrosis of the tsk mouse *in vivo* by more rigorous

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and more dissectable approaches than long-term therapy with multifunctional agents (cromolyn, ketotifen). We reasoned that adoptive transfer of tsk/+ bone marrow stem cells into lethally irradiated syngeneic recipients might provide such a setting. If skin fibrosis were transferable without the characteristic increased numbers and increased degranulation state of the mast cells of tsk/+ skin, our former conclusion that the mast cell is an essential intermediate in the fibrotic process would require reexamination; the same conclusion would apply if mast cell abnormalities, but not skin fibrosis, were transferable. If neither fibroses nor mast cell abnormalities were transferable, the model would hold little promise for future dissection. If both skin fibrosis and mast cell abnormalities were transferable, dissection of the adoptive transfer inoculum might shed light on cell-cell and cell-cytokine interaction relevant to skin fibrosis, the long-term goal of this project. The future use of mast cell deficient allogenic or transgenic syngeneic inocula might also be instructive. As a pilot experiment, we studied the skin fibrotic and pulmonary alveolar consequences of transferring tsk spleen and marrow cells into lethally irradiated syngeneic +/+ mice.

We observed that skin fibrosis is transferable but that the characteristic mast cell abnormalities of the tsk/+ phenotype are not. These preliminary results cast doubt on the essential nature of the mast cell and support adoptive transfer as a suitable model for further dissection of this interesting genetic example of fibrosis.

Materials and Methods

Animals. C57BL/6 tsk/+ and C57BL/6 +/+ mice were obtained from The Jackson Laboratory (Dr. Sid Lane, Bar Harbor, Maine) at 4 weeks of age and housed in the MUSC animal facilities.

Adoptive Transfer. At 5 weeks of age, spleen and bone marrow cells were obtained from 10 tsk/+ mice and 10 +/+ mice. Spleens were removed and cells obtained by extruding spleen tissue through sterile wire meshes into separate petri dishes. Bone marrow cells were obtained by removing the tibia and flushing with a small gauge needle attached to a syringe containing Dulbecco's modified Eagle's medium. Spleen and bone marrow cells were washed, spun at 2000*g* in phosphate-buffered saline reconstituted to a finite volume, and counted. Twenty +/+ mice were irradiated with a GE Maxitron at 15 mA and 250 KEV with a TH3 filter and 15- × 15-cm compression cone at a dose rate of 62.5 rads/min for 11.2 min for a total dose of 700 rads of total body irradiation (to destroy immune cells) and divided into four equal groups. Spleen and bone marrow cells were divided equally and separately injected intraperitoneally in 0.5 ml of phosphate-buffered saline. Group 1 ($n = 5$) received 7×10^6 tsk/+ bone marrow cells, Group 2 ($n = 5$) received 50×10^6 tsk/+ spleen cells, Group 3 ($n = 5$) received 7×10^6 +/+ bone marrow cells, and Group 4 ($n = 5$) received 50×10^6 +/+ spleen cells.

Histology. Mice were housed for 4 months, sacrificed (anesthetized with 0.2 ml of Avertin followed by cervical dislocation), and the skin prepared for histologic observation as follows: A 6-mm punch biopsy of shaved dorsal skin was removed from between the shoulder blades. The cylindrical skin punch was cut in half along its long axis perpendicular to the plane of the epidermis. Each half was embedded with its flat surface down and sections were cut along the long axis of this surface with epidermis nearest the blade. The measurement of the width of the subcutaneous fibrosis layer is carried out in triplicate on each of four sections from each biopsy from each mouse, using an interactive digitizing pad and x-y software as a part of a videoplane image analysis system coupled to a Zeiss photomicroscope (5). For lung histology and morphometry, the trachea was intubated with small tubing attached to a syringe of phosphate-buffered formalin. Lungs were inflated, excised and fixed with formalin at 20 cm of pressure for 24 hr, then processed for light microscopy in the same manner as skin tissues. Pulmonary alveolar sizes were measured on an IBAS Automatic Image Analysis System (Zeiss Obercocken, West Germany). At least 2500 alveoli from multiple sections were counted for each group. The proportion of alveoli of various sizes was calculated as follows: percentage of alveoli = amount of space of each alveolar size divided by total amount of space measured × 100. The presence of tsk/+ alveolar abnormalities in +/+ mice was confirmed by histopathologic observations (to be confident that the computer was not counting *ex vivo* traumatic artifacts).

Results

Hair Loss. Upon sacrifice, hair loss similar to that seen in untreated tsk/+ mice was noted on the dorsal surface of all members of the groups receiving tsk/+ spleen or tsk/+ bone marrow cells; no hair loss was seen in groups receiving +/+ spleen or +/+ bone marrow cells.

Spleen Histology. All spleens from both experimental groups exhibited black necrosis at the pancreatic pedicle, widespread deposits of pigment, sparse to absent plasma cells, and an increased number of germinal centers (45 ± 8/low-power field) than did both control groups (25 ± 4/low-power field); these findings are similar to those seen in untreated tsk/+ mice except for spleen pigment. Melanin and iron stains were negative.

Skin Histology. Groups 1 and 2 (tsk/+ cell recipients) exhibited skin fibrosis similar to that of untreated tsk/+. As shown in Figure 1, animals receiving tsk/+ bone marrow cells and animals receiving tsk/+ spleen showed significant subcutaneous fibrosis (100 and 125 μm, respectively), both less than the fibrotic band width

found in untreated tsk/+ mice (150 μm). Untreated +/+ and +/+ receiving +/+ spleen or bone marrow had one-fourth or less the width of the other three groups (25 μm, 30 μm, 5 μm, respectively).

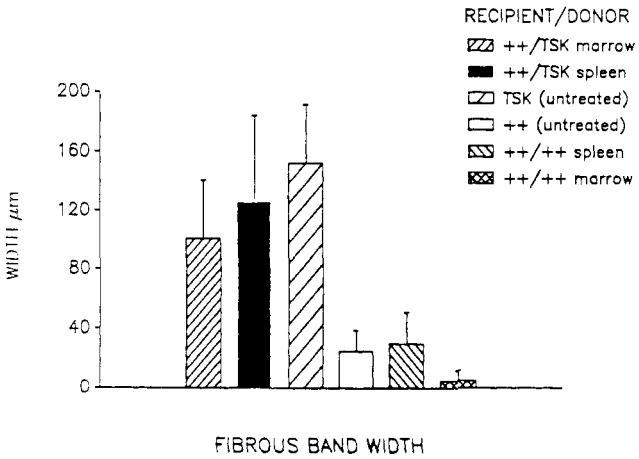

Figure 1. All recipient +/+ mice were irradiated with 700 rads and subsequently injected with cells from tsk/+ or +/+ spleen or bone marrow. Width of subcutaneous fibrous layer was measured in adoptive transfer and control groups. Number of mice studied was 5 +/+ receiving tsk/+ bone marrow, 5 +/+ receiving tsk/+ spleen, 10 tsk/+ untreated, 10 +/+ untreated, 5 +/+ receiving +/+ spleen, and 5 +/+ receiving +/+ bone marrow. Brackets indicate 1 *SD*. Fibrous band width is expressed as mean width in μm ± SD.

Mast cell number and degree of degranulation did not differ significantly comparing +/+ with the four experimental groups, as shown in Table I. Specifically, the characteristic increased number and increased degranulation of mast cells of the tsk/+ mouse were not seen in any of the adoptive transfer recipients, including those who developed skin fibrosis.

Lung Histology. Table II shows that adoptive transfer had a slight effect on airspace size $2 \times 10^4 \mu m$ or larger. Chi-square analysis of all nine categories, as well as of various groupings, revealed three statistically different groups ($P < 0.001$): untreated tsk/+; +/+ receiving tsk/+ spleen and +/+ receiving tsk/+ bone marrow; and untreated +/+, +/+ receiving +/+ spleen, +/+ receiving +/+ bone marrow.

Discussion

The primary finding of this study is the capacity of tsk/+ spleen and bone marrow cells to induce lesions similar in type but milder in degree to those of tsk/+ mice when injected into irradiated +/+ syngeneic mice. A secondary, but no less important, finding is that the skin fibrotic lesions occurred in the absence of detectable abnormalities of mast cells characteristic of the tsk/+ phenotype. The precise level of the immune-inflammatory-fibrotic apparatus affected at which the transplanted cells interact cannot be determined by the

Table I. Mast Cell Density and Degree of Degranulation in Adoptive Transfer of Mice

Degranulation category	Mast cell number/mm² skin					
	Untreated tsk/+	Untreated +/+	+/+ Recipients tsk/+ spleen	+/+ Recipients tsk/+ bone marrow	+/+ Recipients +/+ spleen	+/+ Recipients ++/bone marrow
0	3 ± 1[a]	15 ± 2	17 ± 4	15 ± 4	15 ± 2	13 ± 3
1+	5 ± 4	11 ± 2	12 ± 3	10 ± 2	10 ± 3	10 ± 2
2+	8 ± 3	2 ± 2	2 ± 1	2 ± 2	3 ± 1	2 ± 1
3+	12 ± 3	1 ± 1	1 ± 1	1 ± 0.5	1 ± 1	2 ± 1
4+	40 ± 8	1 ± 0.5	1 ± 1	1 ± 1	2 ± 1	1 ± 1
Total	68 ± 18	30 ± 7	33 ± 10	29 ± 8	31 ± 8	28 ± 6

[a] Number of mast cells/mm² skin in each degranulation state for tsk and +/+ mice immune cell recipients and controls.

Table II. Percentage of Airspace of Different Sizes in tsk/+ and +/+ Mice Immune Cell Recipients and Controls[a]

Airspace size (μm²)	Percentage of airspace					
	Untreated tsk/+	Untreated +/+	+/+ Recipients tsk/+ spleen	+/+ Recipients tsk/+ bone marrow	+/+ Recipients +/+ spleen	+/+ Recipients +/+ bone marrow
1×10^2	25	31	26	24	31	27
5×10^2	25	34	30	31	34	34
1×10^3	18	16	21	20	16	20
4×10^3	12	12	13	12	11	13
8×10^3	9	6	8	9	6	6
2×10^4	7	1	2	3	0.9	0.7
6×10^4	2.0	0.0	0.5	0.3	0.1	0.1
1×10^5	0.7	0.0	0.02	0.03	0.0	0.0
5×10^5	1.4	0.0	0.0	0.0	0.0	0.0

[a] +/+ mice were irradiated with 700 rads and then injected with tsk/+ or +/+ spleen or bone marrow cells. After 4 months, lungs were analyzed for number and sizes of airspaces. With chi-square analysis, untreated tsk/+, control, and experimental groups are each statistically different from the others ($P < 0.001$).

present preliminary observations; these findings do, however, cast doubt on the essential nature of the mast cell in tsk fibrosis. More studies are needed to document the time course of this transfer and to pinpoint the cells involved. The adoptive transfer model is suitable for a variety of potentially instructive *ex vivo* manipulations.

Mast cell densities and the degree of mast cell degranulation in the four experimental groups of mice were similar to those of control +/+ mouse skin and did not show the 2-fold increase in density or the greatly enhanced degree of degranulation seen in the tsk/+ mouse. Whether mast cell products released without degranulation could influence fibrosis remains to be determined. There is precedent for mast cell granule product secretion without degranulation (22).

Both hair loss and fibrosis appear to be transmitted in part by adoptive transfer. Hair loss does not appear to be due to a reduction in follicle number since the number of hair follicles in skin of tsk/+ and +/+ mice is similar (tsk/+ 5 ± 3 follicles, +/+ 4 ± 3 follicles/cm^2 skin). Hair loss could be due to fibrosis affecting perifollicular nutrient circulation.

Spontaneous tsk/+ spleens have many germinal centers that lack B cells (unpublished observations; monoclonal reagents). Possible explanations include abnormalities in B cell differentiation or in B cell homing or both. A variety of immunoglobulin light chain (VK) polymorphisms which could affect B cells or the ability of our monoclonals to detect B cell membrane determinants have been described by Kasturi *et al.* (23). Proliferation of lymphocytes resulting in spleen growth exceeding vascular capacity and resulting in ischemia and necrosis has now been observed in both spontaneous and adoptively transferred tsk studies. Hematoidin-like pigment in the spleens of +/+ mice receiving tsk/+ cells could be accounted for by rapid turnover of tsk/+ lymphocytes with destruction of resident +/+ cell populations and bleeding.

The lungs of +/+ mice receiving tsk/+ marrow and spleen cells showed few very large spaces characteristic of untreated tsk/+ mice but did show a slight increase in airspaces $2 \times 10^4 \mu$m or larger. It should be noted that tsk/+ lung lesions appear in the first 2 weeks of life, earlier than the age of the recipients of adoptive transfer in these experiments (24). The airspace measurements show only slight differences among the various groups, a "soft" finding of unclear significance. A longer incubation time, a larger number of transplanted cells, or younger recipients in future experiments might shed light on the degree and extent of the lung lesions in this model.

From previous reports and the present data, the present model of adoptive transfer is proposed to study the interactions of lymphocytes in the regulation of connective tissue; specific interactions with mast cells and fibroblasts in the skin and with unknown cell types in the lung, perhaps at a different stage of development, could be studied by defining the populations of cells transferred and by varying the age of transfer recipients. Also, tissue-specific subpopulations of transferred cells may be involved in the different pathologic lesions. Further experiments utilizing T cell-depleted transplants could provide additional insight into a possible T cell role in the development of the tsk/+ lesion. Interactions between tsk/+ and mast cell deficient mice might also be of interest.

We have observed adoptively transferable lesions from tsk/+ to +/+ mice with immunocompetent spleen and bone marrow cells, raising the possibility that the immune system plays a role in the initiation of the skin fibrosis of tsk/+ mice. These adoptive transfer experimental results appear to dissociate the skin fibrosis from the increase in number and degranulation of the mast cell, raising the possibility that direct lymphocyte fibroblast signals independent of the mast cell are involved and that fibroblasts and mast cells respond to the same T cell signals. Further study in the tsk/+ model may provide information about possible immune mechanisms in human fibrosis, including human scleroderma.

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